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Spatial-temporal expressions of Crumbs and Nagie oko and their interdependence in zebrafish central nervous system during early development

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Abstract

A vast number of apicobasal polarity proteins play essential roles in the polarization and morphogenesis of the neuroepithelia. Crumbs (Crb) type I transmembrane cell-cell adhesion proteins are among these proteins. Five *crb* genes have been identified in zebrafish. However, their expressional and functional differences during early neural development remain to be fully elucidated. Here, we study the spatial-temporal expression patterns and functions of Crb1, Crb2a, and Crb2b in the central nervous system (CNS) during the neurulation period. We show that: 1, the optic vesicle and undifferentiated retinal neuroepithelium only express Crb2a; 2, Crb1 and Crb2a expressions overlap extensively in the undifferentiated neural tube epithelium; 3, Crb2b expression is the weakest of the three and is restricted to the ventral-most regions of the anterior CNS; and 4, Nok and Crb proteins require each other for their apical localization in neuroepithelium. The commencements of Crb1, Crb2a, and Crb2b expressions follow a spatial-temporal spread from anterior to posterior and from ventral to dorsal and lag behind that of adherens junction components, such as ZO-1 and actin bundles. Genetic and morpholino suppression analyses suggest that in regions where these Crb expressions overlap, they are functionally redundant in maintaining apicobasal polarity of the undifferentiated neuroepithelium.

Keywords

Crumbs; Nagie oko; Lin7c; apicobasal polarity; neuroepithelium

1. Introduction

During neurulation dorsal ectoderm cells reorganize to form the neural tube, which is composed of a single layer of undifferentiated and polarized neuroepithelial cells. The progression of neurulation follows the anterior-to-posterior and ventral-to-dorsal directions (Lowery and Sive, 2004). Both apicobasal and planar polarization are essential for neural tube morphogenesis (Montcouquiol et al., 2006; Copp et al., 2003). The formation and

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maintenance of the apicobasal polarity of neuroepithelial cells require a complex set of apicobasal polarity proteins (Hildebrand and Soriano, 1999; Pujic and Malicki, 2001; Lele et al., 2002; Wei and Malicki, 2002; Murdoch et al., 2003; Lowery and Sive 2005; Hong and Brewster, 2006; Wei et al., 2004; Wei et al., 2006a; Tawk et al., 2007; Munson et al., 2008; Yang et al., 2009). Many of these proteins, such as Cadherin, ZO-1, Lin7c, Crumbs (Crb), Nagie oko (Nok), Pard3, Pard6, Scribble, Shroom, etc., are also required for the proper morphologies and functions of other types of epithelium (Knust and Bossinger, 2002). Eventually, the neural tube differentiates into the complex central nervous system (CNS) (Lowery and Sive, 2004; Colas and Schoenwolf, 2001).

Among these various apicobasal polarity proteins, Crb proteins form evolutionarily conserved protein complexes with Nok and Lin7c homologs (Kamberov et al., 2000; Hong et al., 2001; Bachmann et al., 2001; Hsu et al., 2006; Yang et al., 2009). The Crb proteins are type I transmembrane cell-cell adhesion proteins (Tepass et al., 1990; Zou et al., 2012) and are essential for the apicobasal polarity of many types of epithelium (Izaddoost et al., 2002; Knust and Bossinger, 2002; Makarova et al., 2003; Macara, 2004). The vertebrate Crb homologs can be divided into two groups: the Crb1/Crb2 group, which contain a large extracellular domain, and the Crb3 group, which has a very short extracellular domain (Makarova et al., 2003; Omori and Malicki, 2006). In zebrafish, five *crb* genes have been identified: *crb1*, *crb2a*, *crb2b*, *crb3a*, and *crb3b* (Omori and Malicki, 2006). The *crb2b* gene is transcribed as a longer form (*crb2b-lf*) as well as a shorter form (*crb2b-sf*), with Crb2b-sf lacking the N-terminal half of the extracellular domain of Crb2b-lf (Zou et al., 2012). The structures of Crb1, Crb2a, and Crb2b-lf are very similar and they all have 19-21 EGF-like and three laminin G-like motifs in their extracellular domains (Omori and Malicki, 2006). In situ hybridization analyses indicate that *crb1*, *crb2a*, and *crb2b* genes are expressed in early CNS, *crb3a* in the otic vesicle and digestive system, and *crb3b* only in the digestive system (Omori and Malicki, 2006). However, it remains to be seen whether the temporal and spatial expression patterns of Crb1, Crb2a and Crb2b differ during the development of the CNS.

In this study, we generated antibodies that specifically recognize Crb1. Previously, we have reported the generation of specific antibodies against Crb2a and Crb2b (Zou et al., 2012). With these antibodies, we revealed the distinct yet overlapping spatial-temporal expressions of Crb1, Crb2a, and Crb2b in zebrafish CNS during its early development. These Crb proteins carry redundant polarity-maintaining functions in the regions of the neuroepithelium where their expressions overlap. We also showed that Nok is likely the sole partner that binds to the C-termini of these Crb proteins in undifferentiated neuroepithelium. Furthermore, we showed that proper subcellular localizations of Crb and Nok in the neuroepithelium rely on each other.

2. Experimental procedures

2.1 Animal care

AB wildtype, *nok*^{m227}, *nok*^{m520} and *crb2a*^{m289} zebrafish embryos were raised at 28.5 °C in E3 egg water until desired developmental stages. Care of experimental animals was in accordance with University Pittsburgh guidelines.

2.2 Antibody production

The region of Crb1^{amino acids 952-1258} was PCR amplified with a forward primer (5'gaattcatttcaaacgtgactttcca3') and a reverse primer (5'aagcttcttgcaagtgccagtaa3') and cloned into the His-tag expression vector pET32a+ (Novagen). The Crb1^{amino acids952-1258} region covers the third Laminin A G-like domain and some flanking sequences. The construct was transformed into BL21 competent cells (Invitrogen) and to express the

Crb1⁹⁵²⁻¹²⁵⁸-His fusion protein. Two milligrams of His fusion protein was purified with a His-trap column (Amersham) and used to immunize rabbits (service provided by Proteintech Group, Inc.). Antibodies that recognize Crb1 were affinity purified using an Aminolink Plus immobilization affinity column (Pierce) that was conjugated with 1 milligram of GST-Crb1⁹⁵² fusion protein, which was expressed and purified with the pGex-5x-1 system (Amersham). The generations of antibodies against Crb2a^{amino acids 97-457} and Crb2b-lf^{amino acids 466-773}, Nok, and Ponli were described previously (Wei and Malicki, 2002; Zou et al., 2010; Zou et al., 2012).

2.3 Immunohistochemistry

Crb, Nok, and Ponli antibodies were used at a 1:350 dilution (Wei and Malicki, 2002; Zou et al., 2010). The mouse anti-ZO-1 antibody (Invitrogen) was used at 1:300. The Alexa Fluor 488 Phalloidin (Invitrogen) was used at 1:200. Immunostained sections were examined and photographed as single optical section images under a confocal microscope, which has a maximal axial resolution of 0.8 μ m and an x, y plane resolution of 0.25 μ m (Fluoview FV1000; Olympus, Tokyo, Japan).

2.4 Morpholino knockdown

To block the translation of *crb1*, *crb2a*, *crb2b-lf*, and *crb2b-sf*, about 2 nanogram of each morpholino (Gene-Tools) was injected into each AB wildtype embryo at 1 cell stage. The morpholino was dissolved in distilled water (DNase, RNase free) at 1.5 mg/ml. The sequences of the translation-blocking morpholinos are as follows: *MO-crb1*, 5'CCCAAACCGCTAGATCCATATCCAT3'; *MO-crb2a*, 5'CAGATGCACTTTCCTGATCTCCATG3'; *MO-crb2b-lf*, 5'ACATCCGTCCAAAATCCATGTCCTG3'; *MO-crb2b-sf*, 5'TCACTTTCATATTAGTCCTCTCAT3'. These morpholinos are complementary to regions of the translation start sites of their cognate *crb* mRNA molecules. BLAST searches of the entire zebrafish genomic sequence confirmed that these morpholinos match only to their cognate *crb* genes. In addition, pair-wise alignments of the morpholinos did not reveal significant similarities that would raise the concern of cross-suppression. The specificity of *MO-crb2a* and *MO-crb2b-lf* were previously verified with other splicing-blocking morpholinos (Omori and Malicki, 2006). For triple suppression of Crb1, Crb2a and Crb2b, each *crb* morpholino was dissolved at 1.5 mg/ml, giving a combined concentration of 6 mg/ml. For *ponli* morphants controls, the *ponli* morpholino (gccaacacggtacacgttcagagca) (Zou et al., 2010) was injected at 6 mg/ml.

2.5 Western blotting analyses of Crb expression

The Crb antibodies used in this study are very sensitive for Western blotting conditions, presumably due to their requirement of proper three-dimensional conformation of the extracellular domains of Crb protein antigens. The following special technical details should be integrated into standard Western blotting procedures to detect Crb1 and Crb2a signals: *nok*^{m520}, *crb2a*^{m289} mutant embryos as well as wildtype embryos were collected at 30hpf. The yolks were manually removed with forceps. The de-yolked embryos were homogenized on ice in a lysis buffer at 1.5 μ l/embryo for 30 minutes (1x PBS with 1% Triton X-100, 1mM proteinase inhibitor Phenylmethylsulfonyl fluoride (PMSF), 1 x Roche protease inhibitor cocktail, pH 7.4). The lysates were centrifuged at 14000 rpm at 4 °C for 15minutes to collect the supernatants, which were then mixed with 1/5 volume of 6X SDS loading buffer (375 mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, and 0.03% bromophenol blue), followed by a 5-min incubation at 80 °C (boiling temperature and reducing agents should be avoided to preserve the conformation of the EGF-like domains of the Crb proteins and a brief centrifugation to collect supernatants). The protein extracts from 15 embryos were then

separated on 10% SDS PAGE and transferred to PVDF membranes for blotting analyses. The top halves of the blots were incubated with anti-Crb2a (1:5000) and anti-Crb1 (1:3000) at 4 °C for 20 hrs after a 1-hr of blocking with 5% non-fat milk solution. To detect Crb1 antibodies, the blots were incubated with phosphatase conjugated anti-rabbit IgG (1:3000) for 12hrs at 4 °C. To detect bound Crb2a antibodies, the blots were additionally incubated with mouse-anti-rabbit IgG (1:2000) at RT for 5 hrs, followed by extensive washes and then a subsequent 12-hr incubation with alkaline phosphatase conjugated anti-mouse IgG (1:3000) at 4 °C. For loading controls, the bottom halves of the blots were incubated with α -tubulin antibodies as previously (Zou et al., 2012).

3. Results

3.1 Zebrafish Crb1, Crb2a and Crb2b display distinct and overlapping spatial-temporal expression patterns during neurulation

In order to evaluate the functions and expression patterns of Crb1, Crb2a, and Crb2b in the CNS during neurulation period, we have generated antibodies that specifically recognize distinct regions of the following Crb proteins: Crb1^{amino acid 952-1258}, Crb2a^{amino acid 97-457}, and Crb2b-lf^{amino acid 466-773} (which is also shared in Crb2b-sf) (Zou et al., 2012). Because the Crb2b antibodies cannot distinguish Crb2b-lf from Crb2b-sf, we use Crb2b to represent the two proteins collectively. To ensure that we were comparing the developmental expressions of Crb1, Crb2a and Crb2b in identical regions of the CNS, we focused our comparisons on two CNS regions that can be conveniently defined along the anterior-posterior axis: an anterior region that contains the optic vesicle/cup and the diencephalon, and a posterior region that contains rhombomeres 5 and 6 of the hindbrain that is adjacent to the otic vesicles (Barald and Kelley, 2004).

At the anterior CNS, Crb2a expression was detectable at the apical surface of the optic vesicle as well as of the ventral diencephalon neuroepithelium at 10-somite stage (-ss), whereas Crb1 and Crb2b were not detectable at the same stage (Fig. 1A-C). By 15-ss, Crb2a expression was extended to the dorsal diencephalon (Fig. 1E), whereas Crb1 emerged at the ventral diencephalon neuroepithelium (Fig. 1D). Meanwhile, very weak Crb2b was detected in the ventral-most diencephalon neuroepithelium (Fig 1F). By 20-ss, both Crb1 and Crb2a expressions extended to the apical surfaces of the entire diencephalon neuroepithelium, and Crb2b remained restricted to the ventral-most diencephalon neuroepithelium at a much weaker level (Fig. 1G-I). Meanwhile, Crb2a expression persists at the apical surface of the retinal neuroepithelium, whereas Crb1 and Crb2b were not detectable in the retina at all (Fig. 1G-I).

In the hindbrain near the otic vesicles, none of Crb1, Crb2a and Crb2b was detectable at 10-ss. By contrast, ZO-1 expression was already roughly aggregated at the midline region (Fig. 2 A-C), even though the midline alignment of ZO-1 signals is not as neat and narrow as they are at 15-ss and 20-ss stages (Fig. 2 D-I), illustrating a dynamic ongoing progress of finalizing the apical junctional integrity at this region and time. By 15-ss, Crb1 expression was detectable at the ventral hindbrain neural rod (Fig. 2D), whereas neither Crb2a nor Crb2b was detectable (Fig. 2E-F). By 20-ss, both Crb1 and Crb2a were detectable at the apical surfaces of the hindbrain neural rod (Fig. 2G and 2H), while the expression of Crb2b remained undetectable (Fig. 2I). By contrast, Crb1, Crb2a and Crb2b were not detectable in the otic vesicle at any of the three stages examined (Fig. 2), consistent with previous published in situ hybridization data (Omori and Malicki, 2006). Thus, Crb1, Crb2a and Crb2b are expressed in the CNS with distinct yet overlapping temporal and spatial expression patterns.

3.2 Nok expression patterns resemble the combined patterns of Crb1, Crb2a and Crb2b expression

Crb proteins form complexes with PDZ domain-containing MPP5 homologs through their C-termini (Hong et al., 2001; Bachmann et al., 2001; Hsu et al., 2006). The MPP5 proteins (MPP for membrane palmitoylated protein) constitute a subfamily of the membrane associated guanylate kinase (MAGUK) protein family (Gosens et al., 2008). We thus ask whether the temporal and spatial expression of Nok, a zebrafish MPP5 homolog (Wei and Malicki, 2002), parallels with any of the three Crb proteins in the neural tissue. We found that the expression patterns of Nok resemble the combined patterns of Crb1, Crb2a, and Crb2b expressions, except that Nok is also expressed in the otic vesicles (Fig. 3A-E, compare with Fig. 1 and Fig. 2). This suggests that Crb1, Crb2a, and Crb2b all likely interact with Nok to form functional complexes in the CNS. Supporting this notion, the other of the two known zebrafish MPP5 homolog, Ponli, which is restrictively expressed in green, red and blue cones (Zou et al., 2010; Fig. 3H), is, however, not expressed in the retina, the neural tube, and the otic vesicles at 10-, 15-, and 20-ss (Fig. 3F and G). Because Ponli and Nok are the only known MPP5 homologs in zebrafish, these data suggest that the Nok is likely the sole MPP5 protein that binds to Crb C-termini in undifferentiated neuroepithelium.

3.3 Crb1, Crb2a, and Crb2b appear to carry redundant function in regions where their expressions overlap

Since zebrafish Crb1, Crb2a and Crb2b proteins are very similar in their amino acid sequences and protein domain structures (Omori and Malicki, 2006; Zou et al., 2012), we thus asked if Crb proteins are functionally redundant in the CNS regions where their expressions overlap. To address this question, we utilized specific morpholinos to individually knock down the expressions of Crb1, Crb2a and Crb2b. We found that the loss of function of each Crb protein did not affect the apicobasal epithelial polarity of the diencephalon and the posterior neural rod at the hindbrain region, as evidenced by the smooth apical alignment of apical makers ZO-1 and actin bundles (Fig. 4A-F). The suppression of Crb in these single morphants is homolog-specific because non-cognate Crb homologs were not suppressed (Supplementary Fig. 1). Interestingly, for unknown reasons, about 50% *MO-crb2a* and *MO-Crb2b* morphants displayed otic apical expression of Crb1, which was not observed in wildtype embryos (Fig. 2G; Supplementary Fig. 1C and E). Although loss of individual Crb did not affect the polarity of diencephalon neuroepithelium, when Crb1, Crb2a and Crb2b were knocked down simultaneously with a mixture of morpholinos, apicobasal epithelial polarization of brain neuroepithelium but not otic epithelium was severely affected, as evidenced by misalignment of apical markers ZO-1 and actin bundles in the diencephalon and hindbrain neuroepithelia (Fig. 4G; Fig. 5B-D). This polarity defect was unlikely due to the high concentration of the injected morpholino mixture because the same concentration of *ponli* morpholino did not disrupt neuroepithelial polarity (Supplementary Fig. 2). Although some levels of cell death were observed in both *ponli* and *crb* morphants, the fact that the disruption of cell polarity in *crb* morphants was also observed in non-dying cells, together with the lack of polarity defect in *ponli* morphants suggest that the cellular polarity defect of the *crb* morphants was unlikely a secondary effect of cell death (Supplementary Fig. 2). It is worth noting that Crb2b immunostaining signals were observed in *crb2b-lf* and *crb2b-sf* single morphants; by contrast, Crb2b signals were completely abolished in *crb2b-lf* and *crb2b-sf* double morphants (Fig. 4DF), suggesting both Crb2b-lf and Crb2b-sf are expressed in the neuroepithelium. This is consistent with our previous RT-PCR analyses that showed both *crb2b-lf* and *crb2b-sf* are specifically expressed in the eye at the mRNA level (Zou et al., 2012). Collectively, these data suggest that Crb1, Crb2a and Crb2b play a redundant role in maintaining apicobasal epithelial polarity of

undifferentiated CNS neuroepithelium, and that they do not function in the otic vesicle, which expresses Crb3a (Omori and Malicki, 2006).

3.4 Crb proteins are required for apical localization of Nok in neuroepithelial cells

Because the expression patterns of Nok resemble the combined expression patterns of Crb proteins and because no MPP5 homologs other than Nok have been found to be expressed in undifferentiated CNS (Fig 3), we thus next investigated the possibility of interdependency of Nok and Crb proteins for their apical localization and functions in brain neuroepithelium with Nok or Crb deficient embryos. We found that loss of individual Crb1, Crb2a, or Crb2b did not affect the apical localization of Nok (Fig. 6BD). However, loss of all three Crb proteins largely abolished the apical localization of Nok in the hindbrain neuroepithelium but not in the otic vesicle (Fig. 6E), indicating that Crb1, Crb2a, and Crb2b function redundantly in targeting Nok to the apical surface in the neuroepithelium. Moreover, as shown at higher magnification (Fig. 6F), ZO-1 signals were concentrated as irregular spots (arrowheads) in the apical half of the neural epithelial cells in these triple compound morphants, suggesting the apical basal polarity was not completely lost in these triple *crb* morphants. However, even in the presence of this partial apicobasal polarity, very minimal Nok signals are found to be colocalizing with small number of ZO-1 positive structures (Fig. 6F, arrowheads; also see Fig. 6E, arrows). The Nok signal strength in such places is much weaker than in wildtype neuroepithelium or than in the apical surface of the otic epithelium in the same embryo (Fig. 6E), suggesting that loss of Crb1/Crb2a/Crb2b expression significantly diminished the apical localization of Nok. By contrast, Nok signals appeared to be more diffusely distributed throughout the neural epithelial cells, showing no preferred colocalization with ZO-1 in majority of the morphants neuroepithelial cells (Fig. 6F; arrows indicate basally-localized Nok signals where no ZO-1 signals were detected.). Although we could not state in a quantitative term about the stability of Nok in *crb* triple morphants (also see discussion), these Nok immuno-fluorescence signals suggest the presence of Nok protein in these morphants (Fig. 6E and F). If we could regard the ZO-1 positive structures as residual apical domain of neural epithelial cells in these morphants (Fig. 6F), the above phenomena would suggest that the lack of apical localization of Nok in Crb triple morphants is unlikely a non-specific consequence of the polarity defects; rather, it would suggest that Crb proteins are required for apical targeting of Nok.

3.5 Nok is required for apical localization of Crb proteins in neuroepithelial cells

To examine the role of Nok for Crb localization, we next investigated the distribution patterns of Crb in *nok* mutants. We found that all three Crb proteins lost their apical localization in the diencephalon neuroepithelium in *nok^{m520}* mutants at 26-ss (Fig. 7). However, in these Nok deficient embryos, the polarization of ZO-1 and actin bundles in the diencephalon neuroepithelial cells was largely unaffected; total rounding of neuroepithelial cells was rarely observed and ZO-1 signals were still largely concentrated in the midline vicinity, suggesting that in the absence of Nok, additional layers of polarity maintenance mechanisms, such as adherens junction, are at least partially at play in the brain neuroepithelium. Nevertheless, the apical markers were not smoothly aligned in mutants compared to wildtype and the diencephalon and hindbrain ventricle was not developed as well (Figs. 7). Thus, Nok is required for all three Crb proteins to localize properly to the apical surface diencephalon neuroepithelium at 26-ss.

Interestingly, at later developmental stages, such as at 24 hpf and 36 hpf, when cell division activity slows down in certain brain neuroepithelial regions, certain levels of Crb proteins localize to their apical surfaces in *nok^{m520}* mutants but not in *nok^{m227}* mutants (Fig. 8 for Crb2a; data for Crb1 and Crb2b not shown). Because *nok^{m227}* mutation disrupts Nok's Crb-binding PDZ domain by a single amino acid replacement and *nok^{m520}* mutation truncates its

C-terminal GUK domain and leaves its PDZ domain intact (Wei and Malicki, 2002), the observation of apical localization of Crb at certain brain apical surfaces in *nok^{m520}* but not in *nok^{m227}* mutants has two implications: The first implication is that *nok* mutations do not lead to a complete wipeout of Crb expression during early development stages. This observation, however, does not agree with the conclusion of the complete lack of Crb2a expression in embryonic protein extracts of 30-hpf *nok^{m520}* mutants, which was reached via Western blotting analyses using pan-Crb antibodies that recognize short common amino acid sequences of the intracellular domains of all types of Crb proteins (Hsu et al., 2006). To further verify the presence of Crb1 and Crb2a in *nok^{m520}* mutants at 30 hpf, we also performed Western blotting analyses using our Crb antibodies which specifically recognize the extracellular domains of Crb proteins; we confirmed the presence of Crb1 and Crb2a expression in *nok^{m520}* mutants at 30 hpf (Fig. 8E), suggesting that lack of detection of Crb in *nok^{m520}* mutants by Hsu et al. (2006) was likely due to the low sensitivity of the pan-Crb antibodies. The detection of Crb protein expression in *nok* mutants is not unique to early developmental stages; previously, high levels of Crb proteins were also detected within photoreceptors in *nok* mutants at 4-5 dpf (Wei et al., 2006b; Zou et al., 2012). The second implication of the apical expression of Crb proteins in *nok^{m520}* mutants is that a mutant Nok protein such as Nok^{m520} is still partially capable of targeting Crb to the apical surface of the certain regions of brain neuroepithelium between 24 and 36 hpf, as long as Nok's PDZ domain is still functional.

In summary, the proper apical localizations of Crb proteins in the CNS depend on functional Nok. Our data suggest that Nok is the sole PDZ domain-containing Crb MPP5 partner in the undifferentiated CNS that binds to the C-termini of the Crb proteins. This notion is consistent with the previous observation of direct physical interaction between Nok and Crb proteins in zebrafish (Hsu et al., 2006).

4. Discussion

In this study, we show that the undifferentiated CNS expresses Crb1, Crb2a, and Crb2b in distinct spatial and temporal patterns, with Crb2a and Crb1 expressed more broadly than Crb2b. Specifically, Crb2a is expressed earlier than Crb1 in the diencephalon. By contrast, Crb1 is expressed slightly earlier than Crb2a in the hindbrain region. The optic vesicle and undifferentiated retina express Crb2a alone. Finally, Crb2b expression is restricted to the ventral neuroepithelium in the anterior CNS.

4.1 Spatial and temporal regulation of Crb protein expression for stepwise neural epithelial development

The spatial and temporal divergence of the expressions of different Crb homologs may reflect a requirement for quantitative balance of the overall Crb expression level. Although it is unclear whether the slight temporal variations in the apical accumulation of Crb1 and Crb2a in the diencephalon and hindbrain reflect differences in the timing of their mRNA expressions, protein translation, or protein trafficking, the apical expression of Crb2a but not Crb1 and Crb2b in the optic vesicle and undifferentiated retina should be largely regulated at mRNA expression level because *crb2a* mRNA but not that of *crb1* and *crb2b* is expressed at early developmental stages in the retina (Omori and Malicki, 2006). However, it is yet to know if *crb* mRNA molecules are directly targeted to the apical ends of the epithelial cells to facilitate the subsequent apical location of their translation protein products.

This study reconfirms that the apical localization of adherens junction components such as actin bundles and ZO-1 precede that of the Crb/Nok/Lin7c complex (Fig. 2; Yang et al., 2009). Previously, we showed that temporal regulations of polarity protein expression play important roles in stepwise maturation of neuroepithelial polarity and proper neurulation

(Yang et al., 2009). It would be exciting to generate additional antibodies against various zebrafish polarity proteins to further dissect the maturation process of the neuroepithelium. To this end, with genetic, transgenic and various imaging approaches, the quick and external development of the transparent zebrafish embryos make them a much better system than other vertebrates in order to study how a vast number of apicobasal polarity proteins interplay with each other to regulate neuroepithelial polarity and neurulation.

4.2 Interdependency of Nok and Crb proteins for their apical localizations in epithelia

The interdependency of Nok and Crb proteins for their localizations to the apical surface of the neuroepithelium is consistent with the notions that they are the central components of an evolutionarily conserved protein complex (Macara, 2004), and that their physical association is required for their proper locations and functions. Although they may act as equal partners, and although there is no particular hierarchical relationship between them regarding subcellular localizations and functions, we could not rule out the possibility that one protein is expressed slightly earlier and plays a more upstream role than the other. Unfortunately, our study could not test this latter possibility because our rabbit Nok and Crb antibodies are not suited for such a high-resolution co-localization study.

4.3 Physical stability of Nok and Crb proteins

Unlike what is suggested previously (Hsu et al., 2006), our Western blotting analyses show that loss of Nok function does not cause complete loss of Crb proteins (Fig. 8E). This disparity is likely due to the sensitivity and specificity differences between our Crb paralog-specific antibodies and the pan-Crb antibody used in the Hsu study (Hsu et al., 2006). Although it is relatively easy to confirm the presence of the presumably diffuse distribution of Crb proteins in *nok* mutants by Western blotting analyses, it is impractical to do a similar Western blotting analysis to verify that loss of Crb does not lead to physical degradation of Nok. The reasons for this difficulty are: First, it would be impractical to dissect Crb1, Crb2a, Crb2b expressing tissues from the rest of the body that express Crb3a and Crb3b as well as Nok. Thus, if protein extracts of whole Crb1/Crb2a/Crb2b triple morphants embryos were analyzed by Western, a positive result of Nok signals would not be conclusive. For example, as shown in Fig 6E (arrowhead), Nok apical expression remained strong in the otic vesicle of triple *crb* morphants, positive Nok Western signals would not distinguish whether the signals come from the otic vesicle or from the neuroepithelium. Second, there are five *crb* genes, to completely knockdown all of them (Crb1, Crb2a, Crb2b-lf, Crb2b-sf, Crb3a, Crb3b) with a mixture of six morpholinos for such a Western blotting analysis, the super high concentration of morpholino become toxic to embryos and embryos die before neurulation (already, embryos injected with four morpholinos showed many dying cells Supplementary Fig 2B).

4.4 Differences between retinal neuroepithelium and brain neuroepithelium

Although retinal neuroepithelium is derived from the neuroepithelium of the anterior neural tube, deficiency of Nok or Crb proteins affect apicobasal polarity of the neural retina and diencephalon and hindbrain neuroepithelia differently. As illustrated in Figure 8A and C, apical marker ZO-1 and actin bundles localized ectopically to the interior of the retina at 36 hpf, whereas they largely remained at the apical region in the diencephalon, albeit much less neatly organized than in wildtype (also see Wei and Malicki, 2002; Wei et al., 2006b). Similar observations were also made with *crb2a* deficiency (Malicki and Driever, 1999). It is also interesting that such tissue difference does not emerge until later developmental stages. For example, at 26-ss, retinal polarity remains intact in *nok* mutants (Fig. 7B, D, and F; Zou et al., 2008). Thus, there must be some mechanistic differences in the roles of Nok and Crb in regulating epithelial polarity in different biological contexts in terms of both

space and time. Identification of such differences would provide critical insight into the regulations of epithelial polarity during embryogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Nok	Nagie oko
Crb	Crumbs
hpf	hours postfertilization
CNS	the central nervous system
-ss	-somite stage

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Highlights

- !! Temporal and spatial expression patterns of the Crb1, Crb2a and Crb2b proteins in the early CNS.
- !! Functional redundancy among Crb1, Crb2a, and Crb2b in maintaining the polarity of undifferentiated neuroepithelium.
- !! Interdependency of Crb and Nok in subcellular localizations and polarity maintenance.

Figure 1.

Differential expression of Crb1, Crb2a and Crb2b in the diencephalon and the optic vesicle during early development of the CNS. **A-C.** At 10-somite stage (-ss), only Crb2a (**B**) was detectable at the apical surfaces of the optic vesicle (**B**, arrowheads) and the diencephalon epithelium (arrows), with weaker Crb2a expression in the dorsal diencephalon neuroepithelium than in the ventral region (**B**, arrow). By contrast, Crb1 (**A**) and Crb2b (**C**) expressions were not detectable. **D-F.** At 15-ss, Crb1 expression was detectable at the ventral diencephalon (**D**, arrow), and Crb2a expression was detectable throughout the apical surfaces of the diencephalon (arrows) and the optic vesicle (**E**, arrowheads). Weak Crb2b expression was detected at ventral diencephalon (**F**, arrow). **G-I.** At 20-ss, the Crb1 expression extended to the entire apical surface of the diencephalon epithelium (**G**; arrows), Crb2a expression persisted in the retina (**H**, arrowheads) and the diencephalon (**H**, arrows). Crb2b expression remained restricted to the most ventral region of the diencephalon (**I**, arrow). The ventral diencephalon (VD); dorsal diencephalon (DD); optic vesicle (optic v.); and the retina (R) are marked in panel A and G. Scale bars, 50 μ m.

Figure 2.

The differential expressions of Crb1, Crb2a and Crb2b in the hindbrain at the otic vesicle region during early development. **A-C.** At the 10-ss, Crb1, Crb2a, Crb2b were all not detectable. However, ZO-1 expression (arrowheads) was detectable in the midline region, although not yet neatly aligned. **D-F.** By the 15-ss, Crb1 expression was detectable at the ventral neural rod (D, arrow). By contrast, Crb2a (E), Crb2b (F) expressions were still not detectable. ZO-1 expression (arrowheads) and the actin bundles were more smoothly aligned at the midline region at this stage than at the 10-ss. **G-I.** At the 20-ss, Crb1 (G) and Crb2a (H) were detectable at the entire apical surface of the neural rod (arrows). However, Crb2b (I) expression remained undetectable at 20-ss. The otic vesicle (otic v.) and the neural rod (N) are indicated in panels A, D, G for 10-ss, 15-ss, and 20-ss, respectively. Scale bars, 50 μ m.

Figure 3.

The expressions of Nok and Ponli in the diencephalon, retina and hindbrain during early development. **A.** At 10-ss, Nok expression was detectable in the diencephalon (arrow), and optic vesicle (arrowheads). **B-C.** At 15-ss, Nok expression was detectable in the optic vesicle (arrowheads), diencephalon (arrows), neural rod (double arrows), and otic vesicles (double arrowheads). **D-E.** At 20-ss, Nok expression was detectable in the retina (arrowheads), diencephalon (arrows, D), otic vesicles (double arrowheads) and hindbrain (arrow, E). **F-G.** At 22-ss, Ponli expression was not detectable in the retina, diencephalon, or otic vesicles. **H.** Ponli expression in 3-dpf retina at the region immediate apical to the outer limiting membrane (arrows) was visualized as a positive control for immunohistochemistry of panels F and G. The retina, R; neural rod, N; and diencephalon, D are marked in the merged image panels. Scale bars, 50 μ m.

Figure 4.

Individual loss of Crb1, Crb2a, and Crb2b function did not disrupt the neuroepithelial polarity at 15-ss and 20-ss as evidenced by proper localization of apical makers ZO-1 and the actin bundles (arrowheads). **A-B.** Morpholino suppression of either Crb1 (A) or Crb2a (B) expression did not interrupt the proper apicobasal polarization of diencephalon neuroepithelium at 15-ss. The dashed line circles the ventral brain region where Crb1 should otherwise be expressed in wildtype. **C.** *crb2a^{m289}* mutational deficiency of Crb2a did not affect the apicobasal polarity of the diencephalon (C) at 20-ss. **D-F.** Morpholino suppression of Crb2b-lf (E) and Crb2b-sf (D) or both Crb2b-lf and Crb2b-sf (F) did not affect the apicobasal polarity of the ventral diencephalon neuroepithelium where Crb2b-lf and Crb2b-sf are otherwise expressed (encircled by a dashed line) at 20-ss in wildtype. Arrows indicate the supposed expression of Crb2b-lf and Crb2b-sf in corresponding embryos treated with individual morpholinos against *crb2b-sf* and *crb2b-lf*, respectively. **G.** Simultaneous suppression of Crb1, Crb2a, and Crb2b led to the mislocalization of apical markers ZO-1 (arrows) and actin bundles in the diencephalon at 20-ss. The lack of Crb1, Crb2a and Crb2b immunostaining demonstrated the effectiveness of expression suppression by the morpholinos. “Optic V.” for optical vesicle; Scale bars, 50 μ m.

Figure 5.

Simultaneous suppression of Crb1, Crb2a, and Crb2b severely affected the polarity of the hindbrain neuroepithelium but not that of the otic vesicle. **A.** The combined expression profiles of Crb1, Crb2a and Crb2b in the hindbrain at 20-ss was revealed by using a mixture of Crb antibodies for confocal immunohistochemistry. **B-D.** The suppression of Crb1 (**B**), Crb2a (**C**), Crb2b (**D**) in embryos treated with morpholinos against these Crb proteins was verified with immunohistochemistry using individual antibodies. The polarity of the neuroepithelium is affected in the morphants as evidenced by mislocalization/misalignment of apical marker ZO-1 and actin bundles. By contrast, the apicobasal polarity of the otic vesicle remains unaffected (arrowheads). The lack of Crb1, Crb2a and Crb2b immunostaining demonstrated the effectiveness of morpholino suppression. Scale bar, 50 μ m.

Figure 6.

The localization of Nok and Crb1, Crb2a, and Crb2b relies on each other in the central nervous system but not in the otic vesicle. **A.** Nok localizes to the apical surfaces of the hindbrain neuroepithelium (arrows) and the otic vesicle (arrowheads) in wildtype at 20-ss. **B-D.** Individual loss of Crb1 (B), Crb2b (C), and Crb2a (D) did not affect the apical localization of Nok in the hindbrain (arrows) and the otic vesicle (arrowheads). **E.** Simultaneous suppression of Crb1, Crb2a, and Crb2b disrupted proper apicobasal polarization of the hindbrain neuroepithelium and Nok localized diffusely in the neural rod. By contrast, the apical localization of Nok in the otic vesicle (arrowhead) was not affected. Arrows indicate some occasional ZO-1 positive structures that also show some low level of Nok signals. **F.** A boxed region in panel E was magnified four times to show the biased distribution of ZO-1 in the apical end of the epithelium (arrowheads) compared to the basal ends (arrows). By contrast, there was no clear difference in Nok distribution between apical end and the basal end. "Otic V." for otic vesicle; Scale bar, 50 μ m (A-E) and 12 μ m (F).

Figure 7.

The localization of Crb1, Crb2a, and Crb2b requires Nok in the diencephalon at 26-ss. **A, C, E.** Crb1, Crb2a, and Crb2b localized to the apical surface of the diencephalon (arrows) in wildtype embryos. **B, D, F.** Unlike in wildtype embryos, Crb1, Crb2a and Crb2b could not localize to the apical surface of the diencephalon in *nok^{m520}* mutants. R, retina; D, diencephalon. Scale bars, 50 μ m.

Figure 8.

Crb2a localized to the apical surface of certain regions of the diencephalon neuroepithelium in *nok^{m520}* mutants but not in *nok^{m227}* mutants at 36 hpf. **A, B.** Arrows indicate the local enrichment of Crb2a at the apical surfaces of certain regions of the diencephalon in *nok^{m520}* mutants. A boxed region in panel A is enlarged in panel B. **C, D.** Arrowheads indicate the corresponding apical surfaces of the diencephalon in *nok^{m227}* mutants, showing the lack of apical enrichment of Crb2a. A boxed region in panel C is enlarged in panel D. The apical markers ZO-1 localized ectopically to the interior of the retina (double arrows), as expected in *nok* mutants (A, C). The nuclei were stained with TO-PRO (green). Scale bar, 50 μ m. **E.** Western blotting analyses of protein expression at 30 hpf detected Crb1 in both *nok^{m520}* and *crb2a^{m289}* mutants as well as Crb2a in WT and *nok^{m520}* but not in *crb2a^{m289}* mutants. γ -tubulin blotting was used as loading controls. Please note that even though there are variations in γ -tubulin loading control signals for the Crb2a blotting (likely due to sample loss during preparation), our conclusion that certain level of Crb2a is present in *nok^{m520}* mutant embryos still stands.