Restricted Localization of Ponli, a Novel Zebrafish MAGUK-Family Protein, to the Inner Segment Interface Areas between Green, Red, and Blue Cones

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PURPOSE. The inner segments (IS) of the photoreceptors in vertebrates are enriched with polarity scaffold proteins, which maintain the integrity of many tissues by mediating cell-cell adhesion either directly or indirectly. The formation of photoreceptor mosaics may require differential adhesion among different types of photoreceptors. It is unknown whether any polarity proteins are selectively expressed in certain photoreceptors to mediate differential intercellular adhesion, which may be important for photoreceptor patterning. This study was undertaken to identify such polarity proteins.

METHODS. To identify novel MAGUK-family (membrane-associated guanylate kinase) proteins that are similar to Nagie oko (Nok), the authors performed BLAST searches of the zebrafish genome with the Nok amino acid sequence as the query. The coding sequence of one of the identified genes was obtained and verified through RT-PCR and RACE (rapid amplification of cDNA ends). Its protein expression patterns were examined by immunomicroscopy and Western blot analysis. Morpholino knockdown technology was used for loss-of-function analyses.

RESULTS. The authors cloned a novel nok homolog and designated it photoreceptor-layer-nok-like (ponli). Unlike Nok, which is expressed broadly, Ponli is only expressed at the interface areas between the IS of the green, red, and blue cones in differentiated zebrafish retina.

CONCLUSIONS. Ponli is the first identified polarity protein that is not expressed in all types of photoreceptors. Ponli’s selective distribution stimulates future investigations on its functions for photoreceptor mosaic formation. (Invest Ophthalmol Vis Sci. 2010;51:1738 –1746) DOI:10.1167/iovs.09-4520

In the vertebrate retina, there are two basic classes of photoreceptors: rods and cones. Cones can be further divided into many subtypes. In zebrafish, there are green, red, blue, and UV cones.1 Photoreceptors are polarized cells, with the synaptic terminus, cell body, outer limiting membrane (OLM), inner segment, connecting cilium, and outer segment aligned in the basal-apical direction. Within the plane of the photoreceptor layer, photoreceptors are organized in specific geometric patterns known as photoreceptor mosaics.2 The molecular mechanisms underlying the formation of vertebrate photoreceptor mosaics are unknown.

Genetic studies indicate that the outer limiting membrane (OLM) and the inner segment (IS) are important for maintaining the stability and integrity of photoreceptor layers through lateral cell-cell adhesion.3–5 The OLM and the IS are enriched with many polarity proteins, such as MAGUK-family (membrane-associated guanylate kinase) proteins.5,6 Many of these polarity proteins are expressed in a variety of epithelia, and they either directly or indirectly participate in cell-cell adhesion to maintain tissue polarity and integrity.7,8 It is tempting to speculate that some of these proteins might be expressed in a cell-type-specific manner and may mediate differential lateral adhesion. Such differential intercellular adhesion might underlie photoreceptor mosaics. However, to our knowledge, no studies have thus far revealed any polarity proteins that are expressed in a cell-type specific manner in the photoreceptor layer of the vertebrate retinas.

MAGUK family members are scaffold proteins that contain multiple protein-protein interaction domains. These domains recruit other proteins to form functional complexes. Based on the differences in their domain structures, the MAGUK family is divided into four subfamilies.9,10 Among them is the membrane palmitoylated protein (MPP) subfamily. Thus far, seven types of MPP subfamily proteins have been identified, with erythrocyte p55/MPP1 being its prototype. MPP1 is palmitoylated.11 Palmitoylation is supposed to enhance the association of MPPs to the cell membrane. However, it remains to be confirmed whether palmitoylation occurs in other MPPs. All MPPs contain PDZ, SH3, and GUK protein-protein interaction domains in the N terminus to C terminus direction. Besides these common domains, MPP2–7 contain two L27 domains, and a HOOK domain is also present in MPP1, 2, 5, 6, and 7. Many MPP5s also contain a coiled-coils domain at the N terminus.5 Among them is the membrane palmitoylated protein (MPP) subfamily. Thus far, seven types of MPP subfamily proteins have been identified, with erythrocyte p55/MPP1 being its prototype. MPP1 is palmitoylated.11 Palmitoylation is supposed to enhance the association of MPPs to the cell membrane. However, it remains to be confirmed whether palmitoylation occurs in other MPPs. All MPPs contain PDZ, SH3, and GUK protein-protein interaction domains in the N terminus to C terminus direction. Besides these common domains, MPP2–7 contain two L27 domains, and a HOOK domain is also present in MPP1, 2, 5, 6, and 7. Many MPP5s also contain a coiled-coils domain at the N terminus.5

The nagie oko (nok) gene, which encodes an MPP5 homolog in zebrafish, is required for the integrity of the photoreceptor layers.5 More is yet to be known about the functions of MPPs in the adult retina.

Here we report the cloning and expression pattern of a novel homolog of the nok gene. We designate this gene photoreceptor-layer-nok-like (ponli) for its restricted expression in the photoreceptor layer. Ponli expression coincides with photoreceptor genesis. Unlike Nok’s ubiquitous expression in all photoreceptors, Ponli is expressed only in red, green, and blue cones and is enriched at their IS junctional regions. This unique expression pattern meets the prerequisite for Ponli’s likely function in selective adhesion between these cones.

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METHODS

Zebrafish Care

Adult zebrafish were maintained in a 14-hour light/10-hour dark cycle. Zebrafish embryos were raised at 28.5°C to the desired ages. For immunohistochemical analyses, embryos were first treated with 0.2 mM phenylthiourea in the embryonic day 3 egg water to block melanin pigmentation. Care of zebrafish was in accordance with University of Pittsburgh guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cloning of the ponli Gene

The amino acid sequence of Nok was used as the query to perform the BLAST searches of the zebrafish genome databases at http://blast.ncbi.nlm.nih.gov/Blast.cgi and at http://www.ensembl.org/Multi/blastview. Total RNA, isolated from AB wild-type adult zebrafish eyes with a lysis reagent (Trizol; Invitrogen, Carlsbad, CA), was used to make cDNAs separately with an oligo dT primer or a ponli-specific primer (ggattccaggctgaagggatc). 5' RACE was performed, using primers R1 (cagtcggtctgtccagag) and R2 (gtgagggagccgtacatg) with an amplification kit (GeneRacer; Invitrogen). Attempts of 3' RACE, using primers F1 (gtacatgaggtcaatggaag) and F2 (aggagcgattctccacaatg), did not yield useful information downstream of the stop codon. The RACE products were cloned into pCRII-TOPO (Invitrogen) and sequenced at University of Pittsburgh Genomics and Proteomics Core Laboratories. The GenBank accession number for the nucleotide sequence of the ponli gene is GU197553.

Amino Acid Sequence Comparisons

The amino acid sequences of MPPs were aligned with Clustalw2 (http://www.ebi.ac.uk/Tools/clustalw2/). We used the COILS soft-

FIGURE 1. Ponli is similar to Nok and other MPP5s. (A) Schematic drawing illustrates protein-protein interaction domains of Nok and Ponli. The coiled-coils domain (C-C) locates in the N-terminal conserved (NC) region of Nok. Percentages describe the amino acid similarities between the corresponding domains of Nok and Ponli. (B) Alignment of amino acid sequences reveals extensive conservation at the amino acid sequence level between Ponli and other MPP5s. Black shading: regions of identical amino acids. Gray shading: regions with similar amino acids.
ware (http://www.ch.embnet.org/software/COILS_form.html) to evaluate the presence of potential coiled-coils domains in MPPs. The unrooted phylogenetic relationships between different MPPs were analyzed with VectorNTI and the MEGA4 program (http://www.megasoftware.net/) using the neighbor-joining method. Full-length amino acid sequences of MPPs were used for the analyses.

The following proteins were analyzed. Their accession numbers are in parentheses: human MPP5 (NP_0571919), mouse MPP5 (NP_062525), chicken MPP5 (NP_421200), frog MPP5 (ENSXETT00000024508), fugu MPP5a (ENSTRO00000022797), fugu MPP5b (ENSTRO00000045993), fly Stardust (NP_001053832); rat MPP5 (NP_001101504); orangutans (Q5RDOQ2); chimpanzee (XP0150481); human MPP1 (NP_001024171); human MPP2 (NP_0053565); human MPP3 (NP_0019235); human MPP4 (NP_149055); human MPP6 (NP_7757672); mouse MPP1 (NP_0526471); mouse MPP2 (NP_0579014); mouse MPP5 (NP_0518892); mouse MPP4 (NP_0661252); mouse MPP6 (NP_0645232); mouse MPP7 (NP_001074756); chicken MPP1 (NP_001007918); chicken MPP2 (NP_001253471); chicken MPP5 (XP_418108); chicken MPP4 (XP_001253427); chicken MPP6 (XP_4187212); chicken MPP7 (XP_4185852); zebrafish MPP1 (NP_9998571); zebrafish MPP2 (NP_001002223); zebrafish MPP3 (XP_7008382); zebrafish MPP4 (NP_6852573); zebrafish MPP6 (NP_001038242); zebrafish MPP7 (NP_571051).

### Generation of Polyclonal Anti-Ponli90–386 Antibodies

The cDNA sequence for the region of Ponli from amino acid 90 to 386 was cloned between the EcoRI and XhoI sites of the pET32a+ His-tag expression vector (Novagen; EMD Chemicals, Gibbstown, NJ). The construct was expressed in the BL21 E. coli (Invitrogen). Two milligrams of ponli90–386-His fusion protein, purified from the gel and transferred to nitrocellulose membranes and immunoblotted with rabbit polyclonal anti-Ponli90–386 antibodies (1:1000), rabbit polyclonal anti-Nok28–208 antibodies (1:3000), or mouse monoclonal anti-Nok28–208 antibodies (1:5000; Sigma).

### Photoreceptor Dissociation for Immunohistochemistry

For retinal cell dissociation, we used three transgenic fish lines that express GFP in rods, UV cones, or green/red/blue cones (transgenic fish line strain112; Zou J, et al., manuscript in preparation). Specifically, for each fish line, two retinas were first manually dissociated from the rest of the eye with forceps and were then incubated in 150 µL cell dissociation solution in the presence of amphotericin B and penicillin-streptomycin (TrypLE Express; Invitrogen) for 20 minutes at room temperature. Dissociation incubation was stopped by the addition of 15 µL of (10%) FBS and by chilling on ice. Approximately 150 µL cell suspension was mixed with 50 µL of 2% low temperature agarose in 1× PBS and was spread evenly on glass slides (Thermo Fisher Scientific, Pittsburgh, PA). The cells on the slides were then fixed with 2% paraformaldehyde for 30 minutes and immunostained with the standard protocol.

### Morpholino Injection

Anti-ponli morpholino (gcacacaggtacagcttagagca: Gene Tools, Philomath, OR), which binds to the 5’ UTR of the ponli mRNA, were used to suppress Ponli translation. Morpholino was dissolved in water at 4 nmol/µL and was injected into wild-type AB embryos at the one-cell stage.

### Results

#### Ponli, a Novel Atypical MPP-Subfamily Protein

To identify novel homologs of the nok gene in zebrafish, we performed BLAST searches of the zebrafish genome databases using the amino acid sequence of Nok as the query. The searches revealed a novel gene on chromosome 20. We named it ponli according to its expression pattern. Expression of the ponli gene was validated by RT-PCR and RACE analyses. The

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### Table 1. Identity/Similarity between Ponli and Other MPPs in Four Species

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### Table 2. Identity/Similarity between Ponli and Nok with MPP5s in Other Species

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<td>74/84</td>
<td>76/85</td>
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<td>52/70</td>
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</table>

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Immunostained sections were examined and photographed under a confocal microscope (Fluoview FV1000; Olympus, Tokyo, Japan).

### Immunoblot Analysis

To solubilize proteins, desired tissue samples were homogenized and incubated on ice for 15 minutes in a lysis buffer (1× PBS, 1× protease inhibitor cocktail [Roche, Indianapolis, IN], 1× Triton X-100). Homogenates were centrifuged with a bench-top microcentrifuge at 10,000 rpm for 5 minutes and supernatants were collected and subjected to electrophoresis in 10% SDS polyacrylamide gels. Proteins were transferred from the gels to nitrocellulose membranes and immunoblotted with rabbit polyclonal anti-Ponli90–386 antibodies (1:1000), rabbit polyclonal anti-Nok28–208 antibodies (1:3000), or mouse monoclonal anti-tubulin antibodies (1:5000; Sigma).
gene encodes a 639-amino acid protein that contains two L27N, one PDZ, one SH3, one Hook, and one GUK domain (Fig. 1). This gene structure indicates that Ponli belongs to the MPP subfamily of the MAGUK family.9

We next compared Ponli’s amino acid sequence with the available sequences of MPP1–7 of human, chimpanzee, cow, rat, mouse, zebrafish, fugu (Takifugu rubripes), frog, and chicken in the current genomic databases. The comparison

*ponti* gene encodes a 639-amino acid protein that contains two L27N, one PDZ, one SH3, one Hook, and one GUK domain (Fig. 1). This gene structure indicates that Ponli belongs to the MPP subfamily of the MAGUK family.9

**FIGURE 2.** Phylogenetic analyses of Ponli and other MPPs. (A) Unrooted phylogenetic tree suggests that Ponli is most similar to MPP5s among MPP1–7 in mice, humans, chickens, and zebrafish. However, Ponli is less similar than Nok to other MPP5s. (B) Unrooted phylogenetic tree reveals that Ponli is most similar to the Fugu MPP5b gene among MPP5s of 10 species. The scale bar represents 10% estimated sequence divergence.
indicates that Ponli is most similar to MPP5, with >50% in identity and approximately 70% in similarity (Table 1; data not shown), making the ponli gene the second MPP5 gene in zebrafish besides the nok gene. However, Ponli is less similar than Nok to the MPP5 of other species (Table 2).

Of all the species examined thus far, only fugu has two MPP5 genes, as do zebrafish. Two factors suggest that fugu MPP5b but not fugu MPP5a is more closely related to Ponli, even though fugu MPP5a displays slightly higher percentages in similarity/identity at the amino acid sequence level (Table 2). First, a coiled-coils domain is more strongly predicted by the COILS software in Nok and fugu MPP5a than in Ponli and fugu MPP5b (Fig. 1; data not shown). Second, the results of the phylogenetic analysis using the neighbor-joining method also suggest that fugu MPP5b is more closely related to Ponli (Fig. 2B). These differences suggest that Ponli is not a typical member of the MPP subfamily, implying that Ponli may carry distinct functions compared with its most similar homolog, Nok.

**Restricted Localization of Ponli to the Junctional Regions between the IS of Green, Red, and Blue Cones**

To examine how Ponli differs with Nok functionally, we compared the expression patterns of these two proteins in zebrafish at different developmental stages. To do so, we first generated polyclonal anti-Ponli antibodies, which recognize Ponli but not Nok (Figs. 3, 4). Although anti-Ponli antibodies also recognize two nonspecific bands on Western blot analysis (Fig. 3), they are suitable for characterizing Ponli's expression pattern in the nerve tissue because nonspecific immunoreactivity occurs in the muscle (see Fig. 6D; data not shown).

The expression patterns of Ponli are different from those of Nok. Unlike the extensive expression of Nok in the entire developing and developed CNS and other nonneural epithelia, Ponli is expressed only in the photoreceptor layer of the retina, and its retinal expression does not occur until photoreceptors differentiate (Figs. 3, 4; data not shown). Although Ponli localizes to the ISM in the photoreceptor layer, as does Nok (Figs. 4I–L), Ponli does not appear to be present in all photoreceptors (Figs. 4I–L). To determine whether green, red, and blue cones all express Ponli, we examined Ponli immunoreactivity in dissociated retinal cells. To distinguish among green, red, and blue cones, we used the transgenic fish line...
pt\textsuperscript{112} that was recently established in our laboratory (Zou J, et al., manuscript in preparation). In this line, GFP is expressed in green, red, and blue cones (Fig. 5D, insets). Because zpr1 is only positive in green/red double cones, we counterstained dissociated cells with zpr1 antibodies. Of the 36 cells that were positive for both GFP and zpr1, all were positive for Ponli, indicating that both green and red cones express Ponli because the ratio between red and green cones is 1:1 (Fig. 5C). Of the 19 cells that were positive for GFP but not for zpr1, all were positive for Ponli, indicating that blue cones expressed Ponli as well (Fig. 5D). The absence of Ponli in rods ($n = 44$) and UV cones ($n = 28$) were verified with similar experiments using the rod/GFP and UV-cone/GFP transgenic fish lines (Figs. 5A, 5B). Thus, we concluded that Ponli is expressed in red, green, and blue cones. Ponli’s restricted expression in the junctional regions between the IS of these cones suggests that it plays a different role than Nok in the photoreceptor layer.

**FIGURE 4.** Unlike Nok, Ponli localizes restrictively to the junctional regions between the IS of green, red, and blue cones. (A–D) At 48 hpf, Nok is strongly expressed at the apical surfaces of the retina (arrow) and the brain (arrowhead, C, D), where no Ponli expression (red) is detectable (A, B). (E–H) At 72 hpf, Ponli localizes to the photoreceptor layer (arrow) but not to the apical surface of the brain (E, F, arrowheads), whereas Nok localizes to both regions (G, H). The lack of Ponli signal in the brain indicates that the anti-Ponli antibodies do not cross-react with Nok. (I–L) Imaging of the vertical sections of the retina revealed that Ponli localizes to the IS membrane regions between green, red, and blue cones (I, J) and that Nok localizes to the IS membrane regions of all photoreceptors (K, L). *Arrows:* OLM. (M–P) Tangential imaging of the photoreceptor layer reveals that Ponli localizes restrictively to the junctional regions between the IS of green, red, and blue cones (M, N). By contrast, Nok localizes to the entire circumference of the IS of each type of photoreceptor (O, P). *Arrows:* UV, UV cone; G, green cone; R, red cone; B, blue cone.
Lack of Early Developmental Defects in ponli Morphants

We next tried to determine the function of Ponli in the retina with a morpholino knockdown approach. Immunohistochemistry and Western blot analysis confirmed the specific suppression of Ponli expression in the morphants (Figs. 3B [lane 6], 6). However, loss of Ponli functions did not cause any apparent bodily defects at 3 days postfertilization (dpf; Figs. 6A, 6B). At the cellular/subcellular structural level, photoreceptors appeared normal at 3 dpf (Figs. 6C, 6D). However, we could not determine whether the loss of Ponli would result in photoreceptor defects beyond 3 dpf. This is because the morpholino could not completely suppress Ponli translation later. For example, at 5 dpf, weak Ponli staining was detected by immunohistochemistry, though not by Western blot analysis (data not shown; Fig. 3B, lane 6). Even though transient loss of Ponli function by morpholino knockdown is not sufficient to cause developmental defects in zebrafish, it does not rule out a role of Ponli in photoreceptor maintenance in adulthood (see Discussion).

DISCUSSION

The revelation of Ponli expression in the IS adds complexity to the diverse functions of MPPs in the retina. Recent studies have
revealed MPP1, MPP3, MPP4, and MPP5/Nok in various subcellular regions of the photoreceptors (Table 3). Among these MPPs, MPP5 in mammals and Nok and Ponli in zebrafish are the only ones that localize restrictively to the IS membrane regions (Table 3). However, Nok and Ponli differ from each other significantly. The expression of Nok starts before retinal cell differentiation and plays an important role in maintaining epithelial polarity, retinal cellular pattern formation, and photoreceptor cell layer and RPE integrity.5,16,20 In addition, Nok is also required for the development of other tissues and organs.21–23 By contrast, Ponli expression coincides with photoreceptor differentiation and is exclusively expressed in the junctional regions between the IS of green, red, and blue cones. Thus, Ponli has the narrowest expression scope among all MPPs currently found in the retina.

The unique and intriguing expression pattern of Ponli implies that it may have distinct retinal functions. Two observations prompt exciting speculations about its functions. First, in zebrafish, Ponli-expressing cones have longer IS than Ponli-negative UV cones. The correlation between Ponli expression and the length of the inner segment implies a possibility that Ponli might positively regulate the length of IS in cones. Second, the local distribution of Ponli in the junctional regions between the IS of green, red, and blue cones prompts us to speculate that Ponli is involved in selective intercellular adhesion and, consequently, in photoreceptor mosaic formation.

Two conditions are prerequisite for the formation of geometric photoreceptor mosaics: proper differentiation of different types of photoreceptors and stabilization of specific spatial relationships among photoreceptors. The latter likely requires selective intercellular adhesion. Previously, we demonstrated the involvement of Nok in photoreceptor-photoreceptor adhesion in zebrafish.5 Because Nok is expressed in the entire circumference of the IS of all photoreceptors, Nok-related

![FIGURE 6](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933247/)

**TABLE 3. Subcellular Localizations of MPPs and Their Interactions in the Photoreceptors**

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OPL, outer plexiform layer; CB, cell body; SAR, subapical region; ISM, inner segment membrane; CC, connecting cilium; OS, outer segment.
intercellular adhesion may be important for the overall integrity of the photoreceptor layer but not necessarily for selective photoreceptor-photoreceptor adhesion. However, the restrictive expression of Ponli in the IS of green, red, and blue cones makes it possible for Ponli to mediate selective intercellular adhesion. Unfortunately, our morpholino knockdown experiment did not reveal much about Ponli functions. To gain further insight into Ponli function, other technologies that can block gene expression in zebrafish for an extended period are needed. It would also be important to identify Ponli’s interacting partners and to study how they cooperate with Ponli during retinal development.

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