The Rst-Neph Family of Cell Adhesion Molecules in Gallus gallus


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Abstract: The Rst-Neph family comprises an evolutionarily conserved group of single-pass transmembrane glycoproteins that belong to the immunoglobulin superfamily and participate in a wide range of cell adhesion and recognition events in both vertebrates and invertebrates. In mammals and fish, three Rst-Neph members, named Neph1–3, are present. Besides being widely expressed in the embryo, particularly in the developing nervous system, they also contribute to the formation and integrity of the urine filtration apparatus in the slit diaphragm of kidney glomerular podocytes, where they form homodimers, as well as heterodimers with Nephrin, another immunoglobulin-like cell adhesion molecule. In mice, absence of Neph1 causes severe proteinuria, podocyte effacement and perinatal death, while in humans, a mutated form of Nephrin leads to congenital nephrotic syndrome of the Finnish type. Intriguingly, neither Nephrin nor Neph3 are present in birds, which nevertheless have typical vertebrate kidneys with mammalian-like slit diaphragms. These characteristics make, in principle, avian systems very helpful for understanding the evolution and functional significance of the complex interactions displayed by Rst-Neph proteins. To this end we have started a systematic study of chicken Neph embryonic and post-embryonic expression, both at mRNA and protein level. RT-qPCR mRNA quantification of the two Neph paralogues in adult tissues showed that both are expressed in heart, brain, and retina. Neph1 is additionally present in kidney, liver, pancreas, lungs, and testicles, while Neph2 mRNA is barely detected in kidney, testicles, pancreas and absent in liver and lungs. In embryos, mRNA from both genes can already be detected at as early as stage HH14, and remain expressed until at least HH28. Finally, we used a specific antibody to examine the spatial dynamics and subcellular distribution of ggNeph2 between stages HH20–28, particularly in the mesonephros, dermomyotomes, developing heart, and retina.

Keywords: chicken, immunocytochemistry, IRM proteins, Neph1, Neph2, RT-qPCR

INTRODUCTION

Cell adhesion molecules (CAMs) are key effector elements essential for constructing and maintaining the complex structural and functional organization of the individual organs and tissues in multicellular organisms. Besides their strict adhesive properties, most CAMs are also able to mediate a wide range of dynamic cell–cell and cell–extracellular matrix specific recognition and communication events, both at embryonic and postembryonic stages. These events, in turn, underlie fundamental biological processes such as cell fate specification (Carthew, 2007), neurite growth, and axonal pathfinding (Maness & Schachner, 2007), immune response (Chen & Zhu, 2013), programmed cell death (Zhong & Rescorla, 2012), and epithelial-mesenchymal transition (Gheldorf & Berx, 2013), to name just a few.

A good example of CAM functional diversity is provided by members of the Rst-Neph protein family, a subgroup of the immunoglobulin superfamily evolutionarily conserved from nematodes to humans, whose prototypic member, the *Drosophila melanogaster* Irregular ChiasmC-Roughest (IrreC-Rst), was genetically and molecularly characterized by Karl Fischbach and his coworkers in the early nineties (Boschert et al., 1990; Ramos et al., 1993; Schneider et al., 1995). In flies, IrreC-Rst and its paralogue, Kin-of-Irre (Kirre, also known as Dumbfounded; Ruiz-Gomez et al., 2000; Strünkelnberg,...
et al., 2001), together with their interacting partners, the proteins Sticks and Stones (Sns; Bour et al., 2000) and Hibris (Hbs; Dworak et al., 2001) behave as an exquisitely coordinated, partially redundant protein ensemble, required for the correct implementation of several important developmental processes. These include, among others, myoblast fusion, sensory bristle spacing, ommatidial patterning, neural development (all reviewed in Fischbach et al., 2009), and salivary gland autophagy (Simon et al., 2009).

In vertebrates, three members of this family, Neph1, Neph2, and Neph3 (also known, respectively, as Kirrel1, Kirrel3, and Kirrel2) were identified in mammals and fishes. Their characterization, particularly Neph1, was initially connected with their role of generating and maintaining the structural and functional integrity of the mammalian glomerular slit diaphragm through direct interaction with Nephrin (Sellin et al., 2003; Gerke et al., 2005; recently reviewed by George & Holzman, 2012). Neph1-knockout mice display severe proteinuria with podocyte effacement and perinatal death (Donoviel et al., 2001), although no Neph1 mutations in humans could be identified so far. A mutated form of Nephrin was linked to congenital nephrotic syndrome of the Finnish type in humans (Kestila et al., 1998) and Nephrin knockout mice also show massive proteinuria with perinatal death. Later work showed Neph proteins to be involved also in nervous system patterning (Gerke et al., 2006; Nishida et al., 2010), pancreas development (Rinta-Valkama et al., 2007), myogenesis (Srinivas et al., 2007), and in stromal cell ability to support hematopoietic stem cell survival and keep them in a undifferentiated state (Ueno et al., 2003).

Rst-Neph molecules are single-pass transmembrane glycoproteins, containing either four or five immunoglobulin-like (Igl) domains in their extracellular portion and relatively short, highly divergent intracytoplasmic domains whose main common characteristic is the presence of a PDZ-binding motif at their carboxyl terminus. This feature of the Rst-Neph family, first pointed out in Drosophila Irre-C-Rst (Machado et al., 2002), was shown in mouse to mediate binding of Neph proteins to ZO-1 and PICK1 proteins (Huber et al., 2003; Höhne et al., 2011).

Although Rst-Neph proteins seem to be able to form homodimers, both in vitro and in vivo (Schneider et al., 1995; Gerke et al., 2003), their most common mode of interaction appears to be heterophilic, binding specifically to a family of structurally related proteins, such as the above-mentioned Hbs and Sns of Drosophila C. elegans SYG-2 (Shen et al., 2004) and Nephrin in most vertebrates. This family is similarly comprised of immunoglobulin superfamily glycoproteins with eight Ig-like and one fibronectin type 3 domains in their extracellular portion, a single-pass transmembrane domain and a relatively small and divergent intracellular region. Together, Rst-Neph proteins and their interacting partners appear to define an integrated recognition and signaling platform that is not only conserved but also, to a certain extent, functionally interchangeable across species (Neuman-Haefelin et al., 2010; Helmstädt et al., 2012). Such close functional cooperativity led Fischbach and coworkers to propose the acronym IRM (for Irre Cell-Recognition Module) when discussing these two protein subfamilies (Fischbach et al., 2009).

Different from other vertebrates studied so far, only two Neph homologs, Neph1 and Neph2, are present in chicken (Völker et al., 2012). More intriguingly, Nephrin is lacking, notwithstanding the fact that avian glomeruli appear to be organized very similarly to mammalian ones, with podocytes foot processes connected by typical slit diaphragms (Miner, 2012). These unique features of the IRM in birds make them very attractive organisms to study the Rst-Neph family and their evolution, particularly in the context of their remarkable functional flexibility and general ability to substitute one another.

In this report we have started a systematic expression analysis of Gallus gallus two Neph homologs via quantifying their mRNA levels in the embryo and in adult tissues. We also generated an antibody specifically directed against ggNeph2 protein that allowed us to analyze in detail the dynamics of its spatial distribution in the embryo during organogenesis and suggest its association with actin/adhesive junctions in the myotomes and in the developing eye. Taken together, the data presented here constitute an important prerequisite for further genetic and functional characterization of the Rst-Neph family in chicken.

MATERIAL AND METHODS

Biological Material

Fertilized chicken eggs were obtained from a Brazilian authorized avian products distributor (Granja Yamaguishi, Jaguariuna, São Paulo State) and incubated at 37°C, 28–30% relative humidity until the desired HH stage (Hamburger & Hamilton, 1992) was reached.

Quantitative Real-Time RT-qPCR

Chick embryos and freshly dissected tissues from young adults were immediately transferred into Trizol reagent (Invitrogen) for RNA extraction according to the manufacturer’s specifications. The dried RNA pellets were resuspended in 40 μl of diethylpyrocarbonate-treated water and genomic DNA was removed with DNase I treatment (Promega). The concentration and purity of total RNA samples were determined with NanoDrop spectrophotometer (Thermo Scientific, USA). Only samples with A260/A280 absorbance ratio between 1.8 and 2.2 were further processed for cDNA synthesis.
The High-Capacity Reverse Transcription Kit (Applied Biosystems) was used for the reverse-transcription reaction with the concentration of 2 μg of total RNA sample in accordance with the manufacturer’s protocol. For each target mRNA, 1 μl of each cDNA was amplified in a 15 μl SYBR Green PCR reaction containing 1X PCR Master Mix (Applied Biosystems) and 2 μM of each primer. RT-qPCR reactions were performed in at least two biological replicates, each from a single specimen (embryos) and from a single biological sample (tissues), all in three technical replicates in a 96-well plate (Applied Biosystems) using the Gene AmpH 7500 Sequence Detection System (PE Applied Biosystems). All reactions were run at 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A dissociation curve was run for each plate to confirm the generation of a single product. The relative abundance of ggNeph1 and ggNeph2 mRNA was calculated using the 2^ΔCt method (Livak & Schmittgen, 2001) after the threshold cycle (Ct) was normalized with the Ct of chicken glyceraldehyde-3-phosphate dehydrogenase (ggGAPDH), and also with beta-actin as a second reference gene for the tissue experiments. The Primer Designing tool from the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design specific primers on known sequences. The Primer Express software for Mac (version 6.0d). When a significant value (P < 0.05) was obtained using one-way analysis of variance, further analysis was done. All data showed a normal distribution and passed equal variance testing. Differences between means were assessed using Bonferroni’s multiple comparison test. Data are shown as mean ± standard deviation. Differences were considered significant at P < 0.05.

**In situ Hybridization**

The templates for Neph1 and Neph2 probes were obtained using RT-PCR from embryo cDNA with the primer pairs ggNeph1-F:GACGTGATATGCTGCTCAGG/ggNeph2-R:CGTCTGCCTGGTCTGCTCA (GenBank XM_423078.4 pos.1892–2896) or ggNeph2-F:GCTCGCCTTTCCTGCTCA/ggNeph2-R:TTGCCATCCGGGCCCAAG (GenBank XR_026874.1 pos.91–1076) and cloned into pGEMT. From these, we synthesized digoxigenin-labeled probes by in vitro transcription. Embryos were fixed in 4% paraformaldehyde in PBS for 2 h followed by dehydration in methanol series. Whole mount hybridization was performed at 70°C as described previously (Acloque et al., 2008) and developed with NBT-BCIP substrate after incubation with AP-conjugated anti-digoxigenin antibody. For hybridization in sections, embryos were first rehydrated and embedded in OCT (TissueTek) and 16-μm frozen sections were obtained. Slides were then hybridized at 70°C in a 50% formamide, 10% dextran sulfate, 200 μg/ml tRNA (Roche), 1x Denhardt, 1x salt solution followed by washes in a 50% formamide, 1x SSC, 0.1% Tween-20 solution and staining with AP-conjugated anti-digoxigenin followed by signal development with NBT-BCIP (Acloque et al., 2008).

**Antibody Generation**

Polyclonal antibodies against the chicken Neph2 homolog were generated by initially cloning a cDNA sequence encoding a 118 amino acid-long peptide corresponding to positions 527 to 645 of ggNeph2 (accession number XP_003642655.2. See Supplementary Figure 2 available online at http://informa healthcare.com/doi/abs/10.3109/01677063.2014.933220.) in the E. coli expression vector pQE81 (QIA expressionist-Quiagen). The resulting fusion protein was purified in a Ni Sepharose Fast flow (Amersham Biosciences) column and injected into Albino Wistar rabbits. Immune serum was purified using adsorption to nitrocelulose membranes containing Neph2 fusion peptide and tested in Western Blots (See Supplementary Figure 3 available online at http://informa healthcare.com/doi/abs/10.3109/01677063.2014.933220).

**Immunocytochemistry**

Embryos corresponding to stages HH18, HH20, HH25, and HH28 were collected, washed in PBS, placed in the proper support and frozen in Tissue-Tek OCT. 20 μm sections were then cut using a MicromGmbH D6900 cryostat, placed on gelatinized slides and stored at −20°C until needed. Slides were thawed, then washed in 100 mM Phosphate Buffer pH7.4 for 15 minutes and incubated in blocking solution I (2% BSA) for 30 minutes, followed by another 30-minute incubation in blocking solution II (1% BSA, 2% normal goat serum). For chicken Neph2 immunodetection, sections were incubated overnight at 4°C with anti ggNeph2 rabbit antiserum (26 μg/ml), washed extensively in 100 mM Phosphate Buffer pH7.4 and then incubated, with Alexa Fluor 594 goat anti
rabbit IgG (Molecular Probes; 6 μg/ml) for 2 hours at room temperature. For visualization of cell nuclei and actin cytoskeleton, sections were previously fixed in 4% buffered formaldehyde, washed in phosphate buffer and incubated with Alexa Fluor 488 phaloidin (Molecular Probes; 2.6 u/ml) and DAPI (500 ng/ml) for 30 minutes, room temperature. Sections were mounted in Fluormount, examined and images acquired in a Leica DM6000B light microscope and Leica SP5 Laser Scanning Confocal Microscope.

RESULTS AND DISCUSSION

RNA Quantification of Chicken Neph Homologs in Adult Tissues and During Embryogenesis

Extensive protein database searches were only able to identify two members of the Rst-Neph family in chicken. One of them shows sequence similarity of over 80% to mouse and human Neph1, (Supplementary Figure 1 available online at http://informa.healthcare.com/doi/abs/10.3109/01677063.2014.933220), while the other has over 90% similarity to mouse and human Neph2 (Supplementary Figure 2 available online at http://informa.healthcare.com/doi/abs/10.3109/01677063.2014.933220). These two Neph paralogues show protein similarities of less than 45% between them and of about 40% to mouse or human Neph3. Therefore, throughout the current study we will treat these two sequences as representing the chicken orthologues of mammalian Neph1 and Neph2, respectively.

In order to investigate the spatial and temporal expression dynamics of the two chicken Neph genes, we determined their mRNA levels using quantitative real-time polymerase chain reaction (RT-qPCR), both in different adult tissues and in embryos at developmental stages 14–28 of Hamburger and Hamilton (HH; Figure 1A-D). In adults, Neph1 seems to be the more generally expressed parologue, showing moderate to very high mRNA levels in all tissues examined (Figure 1A) with the exception of testicles, where it is detected at comparatively lower levels. Its mRNA is particularly abundant in kidneys, lungs, pancreas and, to a lesser extent, brain, and liver. Neph2 mRNA on the other hand has a more restricted spatial distribution and a much wider variation of expression levels in adult tissues when compared to its parologue (Figure 1B). While expressed at moderately high levels in brain, retina, and heart, it is either absent or barely detectable in the remaining tissues. Similar differences are found when the embryonic temporal transcription dynamics of the two genes is quantified by RT-qPCR (Figure 1C,D). mRNA from both Neph1 and Neph2 are already present at HH14, but while Neph1 mRNA levels remain essentially constant, at least up to HH28, Neph2 mRNA shows a more tightly regulated profile, rising steadily until HH20, decreasing by HH25, and rising again by HH28.

Overall, the results shown here on the quantification and distribution of ggNeph mRNAs are consistent with data from mammalian Nephs, although important quantitative and qualitative expression differences, particularly in adult tissues, merit discussion. For instance, the extremely high levels of Neph1 mRNA found in pancreas are noteworthy. Although mouse Neph1 transcripts are detected in pancreas by RT-PCR, they do not appear to be translated, as only Neph3 protein is present (Rinta-Valkama et al., 2007). Given the absence of Neph3 in birds, it is tempting to speculate that ggNeph1 could have been recruited to take over some of the functions normally performed by Neph3 in other vertebrates. Another interesting point is the restricted domain of Neph2 RNA expression in chicken, when compared to its mammalian homologs. In mouse, Neph2 protein is present in adult brain, heart, kidney, liver, lung, muscle, and testis (Gerke et al., 2005). Conservation of ggNeph2 mRNA expression in the adult brain was expected, given the critical and highly conserved role of Neph2 homologs in several aspects of brain development and function, (Neuman-Haefelin et al., 2010; Nishida et al., 2011; Prince et al., 2013) including reports of its possible involvement in neurocognitive defects in humans (Bhalla et al., 2008; Guerin et al., 2012). However, its low expression levels in kidney, and absence in lung and liver are intriguing. Clearly, even in the absence of Nephrin and Neph3, the expression patterns of Neph1 and Neph2 in chicken, at least in adult tissues, appear to be more complementary than redundant. This could, in principle, have important implications for understanding the mechanisms that underlie IRM protein function in vertebrates. In Drosophila, functional redundancy between the two Neph homologs IrreC-Rst and Kirre play a very important role both in embryonic myoblast fusion and in the final steps of compound eye patterning (Strunkelnberg et al., 2001; Bao et al., 2010). Furthermore, their transcription, at least in the pupal retina, appears to be coordinately regulated (Machado et al., 2011). The unique organization of IRM in chicken, with only two conserved components, makes this model organism particularly suitable to assess the degree of evolutionary conservation of functional redundancy in the Rst-Neph family.

RNA and Protein Expression Pattern of ggNeph2

Given the relatively complex quantitative and qualitative regulation of chicken Neph2 expression pattern, we decided to undertake a more detailed analysis of the dynamics of its spatial distribution in embryogenesis, both at RNA and protein level. To this end we first performed whole mount in situ hybridization in HH14–HH22
staged embryos, using probes specific for Neph1 and for Neph2 (Figure 2A–H). We refine the expression timeline of Neph1 and Neph2 reported previously, and stress their similarities and differences (Volker et al., 2012). Both Neph1 and Neph2 are detected in the neural tube, otic vesicle and developing retina. The onset of their expression is clearly visible starting from HH14/15. Also, both Neph1 and Neph2 are present in the mesonephric tubules with a relatively weaker signal for Neph2 (Figure 2I–L). In contrast, only Neph2 is expressed in the second brachial arch, limb bud and developing heart. The presence of Neph2 in the heart (Figure 2M) and retina (Figure 2N) at later stages was confirmed with ISH in embryonic slices of stages HH25–28. Here again, Neph1 was detected in the retina in a pattern similar to Neph2 (data not shown), but was not in the heart at the stages analyzed. Together, these results contrast with that of adult tissue expression, in that Neph1 expression during early organogenesis is more restricted than Neph2. Further, they suggest that the embryonic expression of Neph2 is more akin to the adult expression pattern and might play a relevant role in early organogenesis of the heart, retina and brain.

To investigate further this possibility, we checked directly for the presence of Neph2 protein in embryos.
Thus we made a polyclonal antibody for ggNEPH2. The purified antiserum strongly recognized a single of approximately 90kD band in Western blots of lysates from chick embryos staged from HH20 to HH28 (Figure 3A), consistent with the predicted size of about 83kD for the mature protein. To test whether this antibody could also be used for immunodetection in histological slices, we performed immunofluorescent analysis in chick embryo cryosections. The cardiac tissue and the dermamyotome of HH20 embryos presented a strong immunofluorescence signal with very low background staining (Figure 3B). To further confirm the specificity of this antibody we also immunostained embryonic kidneys, since ggNeph2 mRNA has been reported to be already present in developing nephrons from HH20 chick embryos (Völker et al., 2012). Indeed, our antibody-stained nephritic tubules at the distal tip of the mesonephron in HH28 embryos (Figure 4).

Taken together, these data show that the staining pattern generated by our polyclonal antibody supports previous reports of ggNeph2 gene expression pattern and is a reliable tool for detailed analysis of ggNEPH2 protein expression pattern during chick early development. Thus, we performed additional immunodetection analysis of specific tissues during organogenesis.
ggNeph2 in Mesodermal Tissues

The intensity of the immunostaining in HH20 hearts led us to investigate in detail the ontogenesis of ggNeph2 expression during cardiac development. The chick heart derives largely from cephalic lateral mesoderm. After consecutive events of embryonic foldings, the bilateral cardiac tubes are positioned medially where they undergo fusion to form a single pulsating tube. This single cardiac tube is comprised of a primitive atrium and ventricle and will undergo further morphogenesis to position the atrium craniodorsally to the ventricle. At stage HH18 this cardiac looping is finished and the ventricle can be distinguished from the atrium by its thickened cardiac walls and caudal position. It is also at this stage that the ventricular distal part shows the beginning of trabecular formation (Ben-Shachar et al., 1985). Anti-ggNeph2 immunoreactivity was detected in all cardiac tissues, including the trabeculae and remained thus from HH18 through HH28 (Figure 5).

As mentioned above, another tissue prominently labeled was the dermamyotome (Figure 6). This structure arises from the paraxial mesoderm and is destined to become the dermis and muscle segments of the trunk. The paraxial mesoderm—originally a homogenous mesenchymal tissue—progressively condenses into ball-shaped somites that arise periodically following a cranio-caudal order. The somites are then partitioned into the medioventral mesenchymal sclerotome and the dorsolateral epithelial dermamyotome. The former gives rise to the vertebrae while the latter contributes with skin dermis and axial musculature. The development of the skeletal musculature occurs in multiple steps. After primary and secondary formation of the myotome, the muscle precursor cells delaminate and migrate singly or in groups to their final localization (Christ & Ordahl, 1995; Halloway & Currie, 2005). Myotome cells can be first identified by their expression of desmin, vimentin, and alpha-actin (Babai et al., 1990; Ikeda et al., 1968). Alpha-actin appears slightly later than desmin but follows the same striated pattern characteristic of early myotubes (Babai et al., 1990). The pattern of adhesive junctions expression evolves in similarly dynamic fashion. In the spheroidal somite, they abound at the interface between the outer epithelial layer and inner mesenchymal cells (Cinnamon et al., 2006; Duband et al., 1988). Thereafter, they remain enriched in the border between dermamyotome and sclerotome. Finally, when the dermamyotome segregates into dermatome and myotome, only the former maintains high levels of A-CAM and N-Cadherin during myoblast differentiation (Cinnamon et al., 2006; Duband et al., 1988).

Given the intercellular junctional role assigned to Neph2 from previous reports, it would be expected that its chicken homolog would co-localize with adhesive junctions in this tissue. Indeed, ggNeph2 was found in the myotome and displayed a pattern similar to that of previous reports of adhesive junction expression. The ggNeph2 staining pattern was also similar to that of actin (Figure 6B). Thus, considering the strong similarities between f-actin and adhesive junctions staining patterns during the early stages of myotome differentiation analyzed here, we cannot distinguish whether ggNeph2 is associated to adhesive junctions or actin in the myotome.
Figure 5. ggNEPH2 is expressed throughout the embryonic heart. Positive immunoreactivity for ggNEPH2 is detected in all the tissues that comprised both the atrial and ventricular precursor compartments (HH18; A). This expression remained at later stages of development (B,C) and included the growing ventricular trabeculae as well (arrow). At: atrium; V: Ventricle.

On the other hand, the presence of ggNEPH2 in dermamyotome naturally raises the question of the role played by this protein in chick muscle differentiation. In both vertebrate and Drosophila embryos, IRM molecules have been shown to be required for the regulation of myoblast cell fusion. However, the extent of evolutionary conservation of the underlying mechanisms is not clear, especially given the differences in the complexity of myofiber generation in vertebrate and invertebrate systems (summarized in Abmayr & Pavlath, 2012). In Drosophila, the two Rst-Neph proteins IrreC-rst and Kirre function redundantly to mediate migration and association of fusion competent myoblasts with founder cells (Ruiz Gomez et al., 2000; Strunkelnberg et al., 2001). This is an essential step for individual myofiber formation in the fly and depends on heterophilic interactions with Sns (Galleta et al., 2004) and—to a lesser extent—Hibris (Shelton et al., 2009). These interactions, in turn, help trigger a signaling cascade dependent on Arp2/3 complex activation by the SCAR/WAVE (Wip/WASP) pathway that leads to the extensive remodeling of actin cytoskeleton at points of cell–cell contact and allow fusion to proceed (Chen & Olson, 2004; Richardson et al., 2007; Kesper et al., 2007). Vertebrate muscle development is much more complex and less understood “in vivo” than in Drosophila and evidence gathered in zebrafish and mouse, although strongly implicating both Nephrin and Neph proteins in myoblast fusion (Srnivas et al., 2007; Sohn et al., 2009; Durcan et al., 2013), is far from sufficient to provide a general and mechanistically consistent picture of their role in the process. In this context, further study of both spatial and temporal expression dynamics of ggNeph proteins within cell subpopulations derived from myotomic region, as well as comparison with other known mediators of myoblast migration and fusion will certainly contribute to widen our understanding of the precise role of IRM proteins in vertebrate muscle development.

Figure 6. ggNEPH2 is present in dermomyotomes of HH20 embryos (A; white arrowheads). It is particularly enriched in the myotomic domain that stains strongly for actin filaments (B). (C) ggNEPH2 immunofluorescence counterstained with DAPI. Nt: Neural Tube.
Neph2 in the Eye

The adult vertebrate eye is comprised of a distal transparent lens of ectodermal origin and a neural retina and pigmented epithelium derived from the bilateral evagination of the embryonic neuroectoderm. All three components develop in synchrony following highly orchestrated morphogenetic changes and conserved signaling pathways (Chow & Lang, 2001; Sinn & Wittbrodt, 2013). The contact between the bulging retinal anlage with its overlying ectoderm defines the area of the ectoderm that is the lens placode. Thereafter, the optical neuroectoderm folds inward and the lens placode invaginates in concert to form the lens vesicle. Thus, the optical neuroectoderm evolves into a bilayered spherical optic cup with an outer layer of retinal pigmented cells (RPE) apposing an inner layer of retinal precursor cells. Similarly, the lens vesicle is a bilayered structure with a distal layer of proliferating epithelial cells over a proximal layer of elongating lens fiber cells. In both the optic cup and the lens vesicle the bilayered conformations present intense intercellular adhesion at the interface of, respectively, the retina/RPE and the lens epithelia/fiber cells (Duband et al., 1988; Murphy-Erdosh et al., 1994; Sheffield & Fischman, 1970; Xu et al., 2002). The functional importance of these junctions in optic embryogenesis is evidenced during their disruption. For instance, interference with the integrity of intercellular junctions present in the retinal outer layer—also known as the outer limiting membrane (OLM) results in photoreceptor defects (Mehalow et al., 2003).

We observed that ggNEPH2 was highly enriched at the corneal epithelium that overlies the lens vesicle (Figure 7A), the apical membranes of the lens vesicle distal and proximal layers (Figure 7 B,C) and at the retina and RPE contacting surfaces (Figure 7D–I). In the RPE/retina interface we detected the presence of ggNEPH2 less than a day after complete formation of the optic cup (Hamburger & Hamilton, 1992). At HH18 the retina has not yet attained its multilayered histology, and neither have the photoreceptors started their differentiation. Even thus, we observe ggNEPH2 enrichment at the developing OLM. Beta-cadherin expression has been reported to be also heightened at the corneal epithelium and pigmented epithelium at these stages but not in the retina (Murphy-Erdosh et al., 1994) In contrast, A-CAM is present at the same optical sites reported here for ggNEPH2 (Duband et al., 1988).

All these above-mentioned sites are also enriched with actin filaments at the stages we analyzed (Figure 7 J-K) and at later stages as well (Owaribe & Eguchi, 1985; Owaribe & Masuda, 1982; Philp & Nachmias, 1985).

Figure 7. ggNEPH2 is strongly expressed in the eye. Specifically, it is found in the cornea, lens, and retina (A). Analysis at higher magnification of the lens vesicle (B,C,J) and a developmental series of the retinal/RPE interface (D–I) suggest that ggNEPH2 is found mainly at apical membranes with accumulation of actin (J–K). Lens epithelia is upwards (B,C,J) and retina are upwards (D–I). ggNEPH2 (red) immunostaining is counterstained with DAPI (blue) or phalloidin (green).

The Subcellular Localization of cNEPH2 Varies in Different Tissues

Despite their involvement in a large variety of apparently unrelated developmental and physiologic processes, a common theme underlying IRM protein function appears to be their ability to couple cell–cell recognition events with actin cytoskeleton remodeling, upon trans-heterodimer
formation (Garg et al., 2007; Kesper et al., 2007; Simon et al., 2009). The subcellular localization of ggNEPH2 reported here fits well with this general role, as it closely follows the characteristic actin cytoskeleton conformations of the tissues examined. In cardiac tissues, for instance (Figure 8A) ggNEPH2 immunoreactivity mirrors that of actin filaments networks and occurs throughout the cell. In contrast, in the mesonephros (Figure 8B) ggNEPH2 immunoreactivity appears highly enriched at the apical membranes that line the lumen of the nephritic tubules and displays a punctate pattern.

Most interactions between NEPHs and cytoskeleton-associated molecules seem to be mediated by their PDZ binding domain at the carboxyl terminus and in vertebrates these interactions have been characterized almost exclusively in mammalian kidney or brain (Huber et al., 2003; Gerke et al., 2006; Hartleben et al., 2008; Höhne et al., 2011). It is therefore conceivable that further molecular and functional characterization of the role played by ggNeph2 in the tissues examined here could also broaden and refine our understanding of the general mechanisms by which IRM molecules help modulate actin cytoskeleton dynamics during organogenesis. As such, the antibody reported here is a useful addition to the toolbox of the Neph-research community.

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REFERENCES


Supplementary material available online

Supplementary Figures 1–3.