Introduction

The IgSF proteins of Nephrin and Neph families (also called IRM proteins [1]) have been conserved throughout Metazoan evolution. All Nephrin and Neph proteins share extracellular immunoglobulin-like domains and a short cytoplasmic tail that contains multiple signaling motifs [2,3]. The extracellular domains of Nephrin and Neph proteins bind to each other in cis- and/or trans-interactions [4]. Two Neph homologs (IrreC/Rst, Kirre) and two Neph homologs (Hls, Sns) are involved in pupal eye development, muscle fusion and axonal guidance in Drosophila [5–9]. In C. elegans, synapse development and synaptic target recognition also employ members of the Nephrin-Neph protein family. In this system the Nephrin homolog SYG-2 and the Neph1 homolog SYG-1 mediate precise recognition of appropriate partners and trigger synapse formation of the hermaphrodite specific motor neuron (HSNL) [10,11]. Interestingly, all mammalian Neph molecules (Neph1–3) have been shown to be able to functionally replace endogenous SYG-1 indicating a potential redundant function of Neph proteins [12,13]. Despite the diversity of signaling mechanisms and expression patterns of IRM proteins throughout different species, some important themes are beginning to emerge: a striking property of IRM proteins is the formation of variable, homo- and heterophilic interaction modules in cis and trans conformation that precisely guide cellular connections [4,12–15]. An interesting example of such a highly specialized cell-cell contact is the slit diaphragm at the kidney filtration barrier, which consists of cis and trans-interacting Nephrin and Neph1 molecules (Fig. 1C e-h). Mutations in Nephrin lead to congenital nephrotic syndrome which is characterized by a disruption of the kidney filtration barrier, kidney failure and severe protein loss into the urine [16]. In addition, mice lacking Neph1 are proteinuric and reveal effacement of podocyte foot processes [17]. Nephrin and Neph1 molecules have been demonstrated to form a cis- and trans-interacting complex [4,18,19]. Moreover, the Nephrin-Neph1 protein complex has been linked to several signaling processes at the slit diaphragm, like actin regulation, polarity signaling and cell survival [20,21]. Strikingly, recent investigations revealed that Sns and Kirre form a filtration slit in Garland cell nephrocytes (GCNs) of Drosophila (Fig. 1C a-d) which is very similar to the mammalian slit diaphragm of podocytes [15,22,23]. As the experimental accessibility of the mammalian slit diaphragms is very limited, this finding has important implications. Therefore, the Drosophila GCN appears to be an ideal system to study Nephrin-Neph protein functions in a genetically easy tractable system [24]. Interestingly, Sns and Kirre are involved not only in the slit diaphragm formation of GCNs, but also mediate the fusion process of GCNs that results in binuclear GCNs [15].
We employed *D. melanogaster* as a model system to investigate the evolutionary conservation of the Nephrin-Neph proteins and to determine differences between the mammalian Neph proteins 1–3 in their ability to phenocopy and rescue Kirre and IrreC/Rst phenotypes. Our results demonstrate that Neph1 is the only mammalian Neph protein that can mimic the phenotypes of overexpressed Kirre or misexpressed IrreC/Rst in GCNs. Furthermore, we illustrate that only misexpressed Neph1, which partially colocalizes with Sns in GCNs, is able to rescue a GCN fusion phenotype caused by the loss of Kirre. A bioinformatic search for cytoplasmic motifs present in Kirre, IrreC/Rst and Neph1 revealed a conserved 12 AA motif (KIN1 motif), which seems to be required for Kirre and Neph1 function.

### Results

**Overexpression of Kirre and Misexpression of IrreC/Rst, Neph1, but not Neph2 or Neph3, Leads to an Irregular Fusion of GCNs**

A multiple sequence alignment of Neph1–3, Kirre and IrreC/Rst did not reveal a significantly higher sequence similarity for any of the Neph proteins in comparison to Kirre or IrreC/Rst protein (Fig. 1A). In order to investigate differences between the three Neph proteins, we created flies in which transcription of Neph1–3 proteins is under control of the UAS-sequence. We used the GAL4/UAS-System to misexpress V5-tagged versions of the mammalian Neph proteins in different test systems.

During embryonic development mononucleate GCNs fuse to form bimucleate GCNs at the third larval stage (Fig. 2A). The fusion process is mediated by the heterophilic interaction of Kirre and Sns [15]. Overexpression of Kirre driven by *pros-GAL4* resulted in a gain of fusion phenotype at the level of 3rd instar larvae (Fig. 2C). Similarly, the misexpression of IrreC/Rst protein, which normally is not expressed in GCNs [15], resulted in enhanced cell fusion (Fig. 2B). However, out of the three mammalian Neph proteins, only Neph1 proved to induce the fusion phenotype (Fig. 2D). To exclude dose dependent differences the transcript levels of ectopically expressed Neph proteins were quantified by qRT-PCR using primers specific to the V5 tag. This quantitation revealed comparable expression levels of the three Neph proteins with a slightly higher amount of Neph3 mRNA (Fig. S1). The control staining of membrane proteins was not stabilized within the nephrocyte diaphragm are mainly enriched in vesicles due to the high endocytosis rate of GCNs (Fig. 2A). Interestingly, Neph1 colocalized with Sns, suggesting a stabilization in the nephrocyte diaphragm by heterophilic interaction with Sns. Neph2 was partially enriched at cell-cell-contacts without causing enhanced fusion (Fig. 2E); misexpressed Neph3 neither colocalized with Sns, nor did it interfere with the fusion process (Fig. 2F).

**Neph1 Can Rescue the GCN kirre Phenotype**

GCNs express Kirre but do not express IrreC/Rst. Lack of Kirre in *kirre* flies leads to a severe hyperfusion phenotype in the third larval stage that seems to be caused by irregularities during the Kirre-dependent fusion of mononucleon GCNs (Fig. 3A). To further characterize the evolutionary conservation of Neph1 and Kirre at the functional level we used the hyperfusion phenotype for rescue experiments. We found that Neph1 expression using the GCN driver *sna-GAL4* is sufficient to rescue the *kirre* phenotype (Fig. 3). In contrast to this successful rescue experiment, Neph2 or Neph3 expression is insufficient to restore correct GCN fusion. The hyperfused GCNs lose the typically spherical shape and this finding was used to quantify the rescue efficiency (Fig. 3B). These results further support the functional redundancy of Neph1 and Kirre.

**GCN Fusion Requires a Conserved Motif in the Cytoplasmic Tail of Neph Proteins**

An *in silico* search for motifs conserved between IrreC/Rst and Kirre of all *Drosophila* species and Neph1, but not present in Neph2 and Neph3, identified a 12 amino acid sequence which we named KIN1 motif for the proteins in which it occurs (Kirre: PVKFDERFSGDF; IrreC/Rst: PMTLTNSSSGGS; Neph1: PTRFDGPRSSRL) (Fig. 4). To determine the location of this motif in Neph1 we mapped its presence in the recently described structural model of the cytoplasmic domain of Neph1 [25]. Interestingly, this motif was localized in the exposed region of the molecule (Fig. 4C). This further suggests that it is readily accessible to potential interacting proteins and may have a functional role. To validate the importance of the KIN1 motif for the rescue ability of the entire protein, we used cytoplasmically truncated Kirre versions to rescue the GCN phenotype of larva deficient in Kirre. The Kirre versions CT1–CT3 [26], which still contain the KIN1 motif (Fig. 4B), were able to partially restore the wildtype situation. However, Kirre-CT4 lacks the conserved KIN1 motif and was not able to rescue the GCN fusion phenotype, providing evidence for the functional importance of this cytoplasmic Neph1/Kirre motif (Fig. 5).

**Neph1 Misexpression can Mimic Neuronal and Eye Phenotypes of Overexpressed Kirre or IrreC/Rst**

To further investigate the functional redundancy of Neph1 and Kirre we utilized another established model system for Nephrin-Neph function, the *D. melanogaster* compound eye [8,14,27]. As shown previously [1], see-GAL4 induced misexpression of IrreC/Rst or Kirre results in a rough eye phenotype (Fig. 6F and K). Interestingly, misexpression of Neph1, similarly to the misexpression of IrreC/Rst or Kirre, also caused a rough eye phenotype (Fig. 6P). Semithin sections of rough eyes reveal fusion of ommatidia (Fig. 6H, M and R). To quantify the rough eye phenotype, clusters of bristles were counted. These data (Fig. S2) revealed that the severity of the rough eye phenotype in flies misexpressing Neph1 is comparable to flies misexpressing...
Figure 2. IRM protein misexpression in GCNs can induce clustering and/or hyperfusion. A-F. Scanning electron micrograph of *Drosophila* GCNs at third larval stage and immunoreactivity of the corresponding genotypes. The control shows the distribution of misexpressed membrane associated mCD8::GFP. The misexpressed protein is endocytosed if it is not stabilized in the nephrocyte diaphragm. The binucleate GCNs are separated (A). IrreC/Rst misexpression leads to clustering and hyperfusion of GCNs (B). Kirre overexpression leads to a similar phenotype. (C). Neph1 misexpression also leads to clustering and fusion of GCNs (D). Neph2 misexpression does not interfere with the fusion of GCNs (E). The arrow head marks the enriched Neph2 immunoreactivity at cell-cell contacts. Misexpression of Neph3 does not interfere with the GCN fusion. Immunoreactivity shows that the Neph3 expression pattern is similar to the GFP control (F).

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IrreC/Rst. To exclude position effects we tested four lines with independent insertions of the three Neph proteins. Using the driver line Mz1369-GAL4 to drive transgene expression in neurons and glial cells, overexpression of IrreC/Rst resulted in a severe disorganization of all four neuropils of the optic lobe (Fig. 6I). Of these neuropils, the lamina seemed to be least affected, while in some cases the inner optic chiasm between medulla, lobula and lobula plate completely disappeared, leaving the impression of a fusion between these three otherwise clearly distinct neuropils (Fig. 6J). A similar phenotype of variable penetrance resulted from Kirre overexpression (Fig. 6N and O). Strikingly, Neph1 misexpression could phenocopy IrreC/Rst or Kirre overexpression (Fig. 6S and T), whereas Neph2 and Neph3 misexpression had no effect (Fig. S3).

Discussion

The large spectrum of genetic and molecular tools that has evolved for the analysis of protein function in D. melanogaster is unique. As a complex multicellular organism with a sophisticated nervous system and kidney-like slit diaphragm harboring structures, D. melanogaster is an ideal system to study Nephrin-Neph protein function.

While in the C. elegans model all three mammalian Neph proteins are able to partially rescue the synaptic developmental defects of SYG-1 mutants [12], we demonstrate that in D. melanogaster only Neph1, and neither Neph2 nor Neph3, is able to mimic phenotypes produced by either IrreC/Rst or Kirre. This underscores the evolutionarily higher and more complex level of Neph protein function in D. melanogaster compared to C. elegans. Previously, our in silico data indicated that Neph proteins in vertebrates belong to three paralogous groups which probably evolved before or during the emergence of early vertebrates [12]. Such gene duplications often remove the selective pressure from copies, frequently resulting in diverging and non-redundant biological functions of the paralogs. Indeed, our data suggest that, although there are common functions of Neph molecules (as previously evidenced by the ability of all mammalian Neph isoforms to rescue the SYG-1-deficiency phenotype in C. elegans), different mammalian Neph proteins may fulfill rather diverse functions. However, future studies will have to delineate the precise functional diversity of Neph proteins in mammals.

Interestingly, the functional conservation between Neph1 and Kirre throughout metazoan evolution appears to be strong enough for Neph1 to rescue the kirre GCN phenotype. From this finding we conclude that Neph1 is the functional ortholog of the
D. melanogaster Neph-like Kirre. Like Kirre, Neph1 seems to interact with Sns and gets stabilized in the nephrocyte diaphragm. It seems to be a common feature of IRM proteins that overexpression phenotypes and knockdown/knockout phenotypes result in similar phenotypes [1]. In accordance with this, overexpression of Kirre and misexpression of IrreC/Rst and Neph1 in GCNs led to phenotypes similar to those resulting from loss of Kirre, indicating that the fusion process is highly dependent on the correct spatiotemporal expression level of IRM proteins. This also holds true for axonal pathfinding defects where loss and gain of function phenotypes phenocopy each other [9].

In summary, our results suggest that Neph1 shares functional features with Drosophila IrreC/Rst and Kirre which are absent in Neph2 and Neph3 proteins. By searching for motifs conserved in the three proteins Kirre, IrreC/Rst and Neph1, but missing in Neph2 and Neph3, we identified the 12AA KIN1 motif. The unique exposed position of the KIN1 motif at the surface in the three-dimensional structural model of Neph1 places it in an ideal position to interact with other proteins. Both the position of this motif within the sequence and the phylogeny of this gene family suggest that the common ancestor of insects and vertebrates possessed a gene harboring this motif. In the course
of evolution the composition of the Neph protein family C-terminus was probably altered due to genetic insertions and deletions, leading e.g. to loss of the motif in Neph2/3. The fact that Kirre CT4 expression is insufficient to rescue the kirre-phenotype illustrates that the Kirre-dependent GCN fusion is not mediated by adhesion of the extracellular part only. Moreover, it points to an essential role of signaling via the cytoplasmic part for correct fusion, potentially via the KIN1 motif.

The easily tractable and functionally closely related podocyte-like GCN Drosophila system will be a powerful platform for applying further reverse genetic screens identifying regulators and functional domains important for Nephrin-Neph protein trafficking, localization, and signaling.

Materials and Methods

D. melanogaster Husbandry

D. melanogaster stocks were cultured on standard cornmeal molasses agar food and maintained at 25°C unless otherwise mentioned.

Fly Stocks and Generation of Transgenic Flies

The V5-tagged mouse Neph1, 2 and 3 cDNAs were cloned to pUAST vectors [28] and injected into w embryos by the company Genetivision to generate transgenic flies [29]. Insertions were mapped and homozygous strains were generated by repeated crosses with the balancer stock w*; Kr[If-1]/CyO; D[1]/TM6C,Sb[1]/Tb[1]; (1;2;3) (BSN7199). The resulting lines were subsequently crossed to Mz1369-GAL4 (gift from Joachim Urban), sev-GAL4 (gift from Barry Denholm) and sns-GCN-GAL4 (gift from Susan Abmayr). The misexpression results were verified by the use of four different lines for each of the Neph proteins with each of these independent insertions causing a similar phenotype.

The kirre strain Df(1)duf sps-1 contains a small sequence deficiency removing the duf locus only (Prieto-Sánchez et al., in preparation) and was obtained by Mar Ruiz-Gómez via Barry Denholm. Kirre CT mutant flies were kindly shared by Sarada Bulchand.

Immunostaining

Pupal eyes and GCNs (isolated from wandering third instar larvae) were dissected in PBS, fixed for 15 min in 4% parafor-
| X | Sev-GAL4 | Mz1369-GAL4 | Optic lobe drawing |
|---|----------|-------------|-------------------|
| WT | ![Image A](image1.png) | ![Image D](image2.png) | ![Image E](image3.png) |
| UAS-irre/Ct | ![Image B](image4.png) | ![Image G](image5.png) | ![Image F](image6.png) |
| UAS-kirre | ![Image C](image7.png) | ![Image H](image8.png) | ![Image J](image9.png) |
| UAS-neph1 V5 | ![Image L](image10.png) | ![Image M](image11.png) | ![Image O](image12.png) |
maldehyde, wash in PBST (PBS, 0.2% Triton X-100) and incubated with primary antibodies (in PBS, 0.2% Triton X-100, 0.05% sodium azide). A fluorescent dye-coupled secondary antibody (Alexa Fluor 488, Alexa Fluor 568; Invitrogen) was used as the secondary antibody. Preparations were embedded in Vectashield (Vector Labs). The mounting medium for GCNs contained DAPI for nuclear staining. Samples were imaged using NIKON A1 CLEM with inverted microscope Eclipse TI. The following primary antibodies were used: Mouse anti-Roughest Mab24A5.1 (1:10) [9]; rabbit anti-SNS (1:200) [31]; mouse anti-V5 Mab24A5.1 (1:10) [9]; rabbit anti-Kirre A126i (1:200) [30]; mouse antibody (Alexa Fluor 488, Alexa Fluor 568; Invitrogen) was used as the secondary antibody. Preparations were embedded in Vectashield (Vector Labs). The mounting medium for GCNs contained DAPI for nuclear staining. Samples were imaged using NIKON A1 CLEM with inverted microscope Eclipse TI.

Microscopic Examination and Quantitation

GCN preparations and adult eyes for SEM were fixed using Bouin solution. Dehydration with 70% EtOH, 80% EtOH, 90% EtOH and 100% EtOH, was followed by incubation in 50:50 EtOH/HMDS. After incubation in 100% HMDS the solvent was allowed to evaporate. All samples were coated with gold using a Polaron Cool Sputter Coater E 5100. Samples were imaged using a Leo 1450 VP electron microscope. SEM data of GCNs were quantified by measuring the circularity of single nephrocytes after marking their cell shape with Adobe Photoshop. Sections of adult retinae were performed as previously described [33]. Eyes were sectioned through the equatorial region and imaged using NIKON A1 CLEM with inverted microscope Eclipse TI.

Histology

For sectioning, adult flies were fixed, dehydrated, imbedded in paraffin and cut into 7 μm sections as described by Heisenberg and Bohl [1979]. Sections were mounted on coated glass slides, and neuronal structures were visualized by auto fluorescence.

Bioinformatics

Amino acid sequences were aligned using M.A.F.F.T. [34] and the alignment manually curated using Jalview [35]. The most appropriate model was evaluated using ProtTest [36] and turned out to be WAG+I+G+F. Tree reconstruction was carried out using Quicktree SD [37] with 1,000 bootstrap samples and with MrBayes [38] using WAG with eight gamma distributed rates and two hot split frequencies of 0.01. The Bayesian inference and neighbor-joining trees showed the same overall branching order with very minor differences. The Bayesian tree shown was visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Motif detection was carried out using MEME [39] on the cytoplasmic part of the protein sequences, using M. musculus Neph2/3 as negative and M. musculus Neph1 as well as Drosophila Kirre and IrreC/Rst and D. rerio Kirre-like sequences as positive set. Using a manually curated alignment of M. musculus Neph, D. rerio Kirre-like and D. melanogaster Kirre and Roughbest sequences, two motifs were confirmed to be present in Neph1 and Kirre/IrreC/Rst, but not in Neph2/3. The molecular modeling of the cytoplasmic domain of Neph1 was performed as recently described [25].

RNA Isolation from 3rd Instar Larvae and Quantitative RT-PCR

Whole 3rd instar larvae were homogenized in Trizol using an Eppendorf homogenizer. RNA was purified with chloroform followed by DNase treatment. The design of V5-tag specific primers was carried out using the public software primer3 (Primer Selection Program http://fokker.wi.mit.edu/primer3/input.htm). cDNA was synthesized using a mixture of oligoT and random hexamer primers from 0,5 μg of total RNA using Superscript II (Invitrogen) following the manufacturer’s protocol. Real time PCR was performed for 45 cycles using Lightcycler 480 (Roche). The log2 ratios (transgene/wild-type fly) were calculated as described [40]. The actin gene was used for normalization. Individual PCR amplifications were carried out in duplicates and analyses included three biological replications.

Supporting Information

Figure S1 Expression level quantitation of Neph1, Neph2 and Neph3. The amount of Neph1, Neph2 and Neph3 mRNA was quantified by qRT-PCR. Results are presented as log2 ratio of CTP values obtained under transgene misexpression and control. Samples were normalized using the actin gene. The transcript levels are in a comparable range. The amount of Neph3 mRNA is slightly higher than Neph1 and Neph2 mRNA. Genotypes: pros-GAL4/UAS-Neph1_V5, pros-GAL4/UAS-Neph2_V5 and pros-GAL4/UAS-Neph3_V5. N = 3. (TIF)

Figure S2 Rough eye phenotype quantitation. To quantify the rough eye phenotype, clusters of bristles were counted. The severity of the rough eye phenotype in flies misexpressing Neph1 is comparable to that in flies overexpressing IrreC/Rst. For each Neph protein data of four independent insertions are shown. Arrows are marking the lines selected for the rescue experiments and qRT-PCR. (TIF)

Figure S3 Neph2 and Neph3 cannot mimic neuronal and eye phenotypes of overexpressed Kirre or IrreC/Rst. Scanning electron micrographs of adult Drosophila eyes (A,B,F,G,L,Q) and light micrographs of semithin sections (C,H,M,R). The control shows the regular crystal like arrangement of an Drosophila eye (A,B). Sev-GAL4 induced misexpression of IrreC/Rst (F,G) or Kirre (K,L) results in a rough eye phenotype. Misexpression of Neph1 with sev-GAL4 also causes a rough eye phenotype (P,Q). All three genotypes exhibit fusion of ommatidia (H,M,R). Genotypes: sev-GAL4/+ (A,B,C); sev-GAL4/UAS-irreC/rst (F,G,H); sev-GAL4/+UAS-kirre/+ (K,L); sev-GAL4/UAS-neph1_V5 (P,Q,R). Auto fluorescence micrographs of adult Drosophila optic lobes (D,I,N,S). The control fly shows the typical wildtype-like arrangement of the neuropils of the Drosophila optic lobe (D,E). Overexpression of Rst (I) or Kirre (N) causes severe misrouting of fibers in medulla and lobula complex and a disorganization of these neuropils (J,O). Misexpression of Neph1 leads to a similar phenotype (S,T). Optic lobe drawing: la: lamina, me: medulla, lo: lobula, lp: lobula plate. Genotypes: Mz1369-GAL4/+ (D); Mz1369-GAL4/UAS-rst (I); Mz1369-GAL4/+UAS-kirre/+ (N); Mz1369-GAL4/UAS-neph1_V5 (S). doi:10.1371/journal.pone.0043030.g006
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Author Contributions

Conceived and designed the experiments: MH MG KFF TBH. Performed the experiments: MH. Analyzed the data: MH KL ADN SAR MS KFF TBH. Contributed reagents/materials/analysis tools: MG MS SAR KFF TBH. Wrote the paper: MH TBH.

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