Peripherin-2 couples rhodopsin to the CNG channel in outer segments of rod photoreceptors

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Received May 5, 2014; Revised and Accepted June 19, 2014

INTRODUCTION

Outer segments (OSs) of rod photoreceptors harbor all essential proteins of the phototransduction cascade including the G-protein-coupled receptor rhodopsin, the guanylyl cyclase, the phosphodiesterase and the rod cyclic nucleotide-gated (CNG) channel. Most of these proteins localize to highly structured intracellular membrane stacks (disks), which are physically separated from the plasma membrane (1). One exception is the CNG channel, which is exclusively found in the plasma membrane (2). Moreover, the periphery of disks facing the plasma membrane, referred to as disk rims, contains a set of proteins that differ from that of the remaining part of the disks. Previous studies revealed that the rod CNG channel is directly connected to the disk rims via a direct protein–protein interaction of the glutamic acid-rich (GARP) domain of the CNGB1a subunit and the disk rim-specific tetraspanin peripherin-2 (3,4). Peripherin-2 is a scaffolding protein reported to be responsible for the disk morphogenesis and for membrane fusion processes (5,6). Mutations in peripherin-2 are associated with severe retinal diseases (http://www.retina-international.org last accessed 26 June 2014). The vast majority of these mutations are linked to autosomal dominant retinitis pigmentosa (adRP), a degenerative retinal disease that affects rod photoreceptors and often results

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in complete blindness. Here, we combined in vivo and in vitro biochemical, imaging and immunoaffinity methods to show that peripherin-2 in rod OSs binds to rhodopsin and the CNGB1a subunit of the CNG channel. Moreover, we demonstrate that the fourth transmembrane domain (TM4) of peripherin-2 is essential for its interaction with rhodopsin and that a single adRP-associated point mutation in TM4 of peripherin-2 abolishes this interaction while leaving the binding to CNGB1a unaffected. These results indicate that peripherin-2 operates as an adaptor protein in the disk rims that physically couples the rod CNG channel to the phototransduction cascade. This physical coupling between rhodopsin and the CNG channel suggests that phototransduction in rod OS is more spatially confined than it has been known so far and could help to gain new insights on the functional and structural role of the disk rim microcompartment in the rod OS. Finally, we conclude that the adRP phenotype in patients carrying a mutation in TM4 of peripherin-2 most probably results from the specific disruption of the peripherin-2/rhodopsin interaction in the disk rims.

RESULTS

Identification of CNGB1a-interacting proteins

To identify novel components of the rod photoreceptor, CNG channel complex of retinal lysates of light-adapted 6-week-old-mice were immunopurified using an antibody directed against the C-terminus of the CNGB1a subunit of the rod CNG channel and analyzed by quantitative mass spectrometry. Lysates of age-matched, CNGB1-deficient mice served as negative control. Among the list of OS-specific CNGB1a-interacting proteins, we identified rhodopsin and peripherin-2 (Supplementary Material, Table S1). Reciprocal co-immunoprecipitations (co-IPs) from retinal lysates using either an anti-rhodopsin antibody (Fig. 1A) or an anti-CNGB1a antibody (Fig. 1B) confirmed that rhodopsin, peripherin-2 and CNGB1a are present in the same channel complex. Importantly, no specific bands were observed when performing the co-IPs with retinal lysates from CNGB1-knockout mice (Fig. 1B, lane 2) or when a control IgG was used instead of the anti-CNGB1a antibody (Fig. 1A, lane 2). In addition to monomeric rhodopsin, the anti-rhodopsin antibody detected bands corresponding to rhodopsin oligomers (i.e. dimers, trimers and tetramers) pointing to a very tight interaction between rhodopsin molecules, which persisted under SDS–PAGE conditions. To get a more direct view on the localization of rhodopsin and CNGB1a in rod OSs, we employed immunoelectron microscopy in retinal sections (Fig. 1C). Immunogold particles of different diameters co-labeled with the anti-rhodopsin and anti-CNGB1a antibodies, respectively, revealed that CNGB1a is localized in close proximity to rhodopsin.

Analysis of the rod opsin/peripherin-2/CNGB1a complex in HEK293 cells

In previous studies, it was shown that peripherin-2 binds to the N-terminal GARP domain of CNGB1a (3,4). However, an interaction between peripherin-2 and rhodopsin or between rhodopsin and CNGB1a has not been reported so far. To address this issue, we performed co-IPs in HEK293 cells expressing different combinations of the chromophore-free rod apo-opsin (herein referred to as rod opsin), peripherin-2 and the rod CNG channel subunits (Fig. 2A and B). Surprisingly, in the absence of peripherin-2, rod opsin did not assemble with CNGB1a nor did it interact with this subunit when both subunits of the native CNG channel (CNGB1a + CNGA1) were present (Fig. 2A). In contrast, when peripherin-2 was co-expressed together with rod opsin and CNGB1a, the CNGB1a subunit could be co-immunoprecipitated with the anti-rhodopsin antibody (Fig. 2B). Moreover, peripherin-2 was binding to rod opsin in the absence of CNGB1a (Fig. 2B). Taken together, the co-IP experiments indicate that rod opsin requires peripherin-2 to interact with CNGB1a. We therefore postulated that peripherin-2 physically links rod opsin and CNGB1a by simultaneously binding to both proteins.

To test this hypothesis, we applied fluorescence resonance energy transfer (FRET) that allows detection and quantification of protein–protein interactions (Fig. 2C). We tagged the proteins C-terminally with either citrine or cerulean and then used the respective fusion protein pairs for FRET measurements. In agreement with the immunoprecipitation data, we obtained robust FRET signals for rod opsin/peripherin-2 and as well as for peripherin-2/CNGB1a. Quantitatively, FRET ratios (FRs) of these pairs were in a similar range. However, they were somewhat lower than FRs obtained for rod opsin or peripherin-2 homodimers (see also Fig. 1A and B). In agreement with previous findings (3,4), our FRET data also revealed a robust interaction between peripherin-2 and the soluble GARP2, which corresponds to the N-terminal portion of CNGB1a. Importantly, the FR obtained for the rhodopsin/CNGB1a FRET pair was only slightly above background confirming that there is no specific interaction between these two proteins.

In vitro FRET unveils binding characteristics of rod opsin and peripherin-2

To narrow down the region in peripherin-2 that is required for the interaction with rod opsin, we determined the FRs between the cerulean-tagged rod opsin and citrine-tagged peripherin-2 harboring C-terminal truncations (Fig. 3A). While deletion of the C-terminus downstream of the fourth transmembrane segment (Prph2_C1-citr) had no effect on the interaction with rod opsin, additional truncation of the TM4 domain (citr-Prph2_C2) abolished binding to rod opsin (Fig. 3A). In the latter truncation mutant (containing only three transmembrane segments), citrine was fused to the N-terminus to ensure that (like in C-terminally tagged peripherin-2 variants, containing four transmembrane domains) citrine faces the cytoplasmic side of the cell. Importantly, the position of the tag did not interfere with the principal interaction with rod opsin because FRs of the peripherin-2/rod opsin pairs were in a comparable range for either N- or C-terminally tagged peripherin-2 (Fig. 3A).

In order to specifically examine protein interactions in the plasma membrane and to determine binding curves of the rod opsin/peripherin-2 interaction, confocal FRET experiments were performed in HEK293 cells (Fig. 3B and C). Maximal FR values (FRmax) obtained with this approach for rod opsin/peripherin-2 and rod opsin/rod opsin pairs, respectively, were in good agreement with the FR obtained in standard (non-confocal) FRET (Fig. 3A). As expected, confocal FRmax values were consistently higher than the corresponding...
Figure 1. CNGB1a, peripherin-2 and rhodopsin are assembled in a protein complex in rod OS. (A) Retinal lysates of wild-type (WT) mice were immunoprecipitated with an anti-rhodopsin antibody (α-Rho) or a nonspecific control IgG (α-IgG). Precipitated samples were analyzed in immunoblots (IB) using α-Rho and antibodies directed against the CNGB1a subunit (α-B1a) and peripherin-2 (α-Prph2) as indicated. (B) Left, retinal lysates of WT and CNGB1-knockout mice (B1−/−) were immunoprecipitated with α-B1a and probed with α-Rho, α-B1a and α-Prph2. Right, loading control containing 10% of the protein lysate used for the co-IP in the left panel. (C) Immunoelectron microscopy images of longitudinal (left) and transversal (right) sections of rod OS co-stained with small-diameter immunogold particles coupled to α-B1a and large-diameter particles coupled to α-Rho, respectively. Co-localization of large and small particles is marked by arrows. Scale bar, 100 nm.

FRET measurements in isolated rod OSs

To demonstrate that rhodopsin is bound to peripherin-2 in native rod photoreceptors, FRET constructs were exprssed in the murine retina using AAV8-vectors, which contain the human rhodopsin promoter (Fig. 4A). Three to four weeks after subretinal injection of viral vectors, robust expression of individual constructs was observed (Fig. 4B). The expression was restricted to rod OSs indicating that the fluorophores did not interfere with the ciliary transport (Fig. 4B). OSs isolated using a self-designed quick protocol appeared to be intact with regard to their structure and shape (Fig. 4C). Figure 4D shows co-expression of a representative FRET pair (rhodopsin-cerulean and peripherin-citrin) in an isolated rod OS. As expected for a native system, FRs were throughout smaller as found upon heterologous expression in HEK293 cells (Fig. 4E). This difference most probably can be ascribed to the fact that the unlabeled wild-type proteins in vivo interfere with the FRET constructs leading to the reduction of the absolute FRET signal. Qualitatively, however, results in rod OSs were consistent with the results in HEK293 cells. Highest FRs were observed for the rhodopsin homomer. Importantly, there was also clear FRET between rhodopsin and peripherin-2 with the FR being slightly higher for the N-terminally as compared with the C-terminally tagged peripherin-2. In contrast, in good agreement with previous studies (4,7), no FRET was observed for rhodopsin and rod-specific GARP2 protein.

An adRP-linked mutation in TM4 of peripherin-2 disrupts binding to rhodopsin

As mentioned before, mutations in peripherin-2 have been associated with adRP. Thus, we were wondering whether some of these mutations may lead to an impaired rhodopsin binding. To address this issue, we analyzed whether some adRP-linked mutations are localized in TM4 of peripherin-2, the domain that we found to be crucial for binding to rhodopsin (cf. Fig. 3A). Indeed, one adRP-associated mutation (p.G266D) (8) was found in TM4 and was located in the transmembrane region most proximal to the intradiskal portion of peripherin-2 (Fig. 5A). Consequently, we tested whether this mutation interferes with the binding of peripherin-2 to rhodopsin. Using FRET (Fig. 5B) and co-IP (Fig. 5C) experiments from HEK293 cells, we demonstrate that the p.G266D mutant abolishes binding to rod opsins. To further demonstrate the specificity of this finding, we analyzed the effects of two additional peripherin-2 mutations on rhodopsin binding. Interestingly, the exchange of the glycine residue at position 266 and glutamate at position 276 by the neutral amino acid alanine (p.G266A and p.E276A, respectively) did not affect the binding to rhodopsin (Fig. 5B and C). Importantly, the interaction of single peripherin-2 mutants with CNGB1a was not affected as shown by additional co-IP experiments from HEK293 cells (Fig. 5D). This indicates that the lack of binding of the p.G266D mutant most probably is not due to its overall folding deficiency. Notably, for co-IP experiments, we used membrane preparations, and in the respective input controls, we could not observe any difference in the membrane expression levels of the mutants compared with the wild-type peripherin-2 (Fig. 5C and D, input controls), which argues against the lack of the membrane expression or transport deficits for any of the mutants analyzed.

DISCUSSION

In this study, we show that peripherin-2 forms a stable protein complex with both rhodopsin and the rod CNG channel in rod OS. Several lines of evidence support this conclusion. First, antibodies specific for the rod CNGB1a subunit immunoprecipitated rhodopsin from murine retinal lysates. Similarly, immunoprecipitation with rhodopsin-specific antibodies pulled down CNGB1a. In both sets of experiments, peripherin-2 was
identified in the immunoprecipitated complex. Experiments in HEK293 cells revealed that rhodopsin does not directly bind to the CNG channel but rather requires peripherin-2 as a molecular linker to form a complex with the channel. FRET experiments strongly supported this ‘bridging’ function of peripherin-2. Based on our confocal FRET measurements, the relative binding affinity for the peripherin-2/rhodopsin interaction was in the similar range as for the rhodopsin/rhodopsin homomer. To the best of our knowledge, the absolute binding affinity of rhodopsin homomers was not determined so far. However, in our co-IP experiments, rhodopsin dimers and oligomers could be detected even under stringent SDS–PAGE and reducing conditions. Based on this, we expect the absolute binding affinity of rhodopsin homomers to be rather high. These results indicate that rhodopsin binds to peripherin-2 with a high affinity that is comparable with that of the rhodopsin homomer. Our biochemical and FRET experiments in isolated OSs strongly support the presence of rhodopsin dimers and oligomers in the native tissue. This is in line with recent studies providing strong evidence for the existence of rhodopsin dimers and oligomers in the native environment (9–11). In this study, we also identified the molecular determinants required for the rhodopsin/peripherin-2 interaction. As the major portion of both proteins is residing in the disk membrane of rod OS, it seems reasonable to assume that the interaction occurs via the transmembrane helices. In support of such a model, deletion of transmembrane domain 4 in peripherin-2 abolished binding to rhodopsin. Moreover, a single glycine to aspartate exchange at the position 266 in the TM4 of peripherin-2 that was previously reported in patients suffering from adRP (8) resulted in loss of binding to rhodopsin. Importantly, binding to CNGB1a was unaffected in the G266D mutant suggesting that the pathophysiological impact of this mutation relies on the impairment of the interaction with rhodopsin. The functional role of intradiskal and intracellular domains of peripherin-2 was examined in numerous studies (12). However, to our best knowledge, only one study addressed the role of a transmembrane domain of this protein showing that the glutamate residue at position 276 in TM4 was crucial for disk morphogenesis (13). Interestingly, the exchange of this charged amino acid to alanine (E276A) did not affect the interaction with rhodopsin. Similarly, the G266A mutation also had no effects on the peripherin-2/rhodopsin complex formation. These results suggest that introduction of charged residues on position 266 disrupts the binding of peripherin-2 to rhodopsin.
whereas neutral amino acids at the same position do not affect this interaction. Taken together, our data point to a key role of TM4 for the peripherin-2/rhodopsin interaction and suggest that impaired binding to rhodopsin may contribute to the pathophysiology of peripherin-2 mutations.

As is evident in Figure 5C and D, two bands for wild-type peripherin-2 are detected in the inputs and co-IP experiments. The upper band of peripherin-2 is most likely not a result of glycosylation because our protein samples were deglycosylated prior to the SDS–PAGE. Intriguingly, the relative intensities of the two bands correlate with the number of negative charges in TM4. In the G266D mutant that contains two negative charges (E276 and D266), the upper band is stronger than the lower band. In contrast, in G266A and wild type containing one negative charge in TM4 (E276), the lower band is stronger than the upper band. Finally, in the E276A mutant containing no negative charge, only the lower band is visible. A potential effect of charges on the conformation and relative mobility of peripherin-2 would be in agreement with previous findings showing that single mutants can lead to a differential electrophoretic mobility of the corresponding protein (14).

Finally, we used FRET and immunoelectron microscopy to directly demonstrate that rhodopsin and CNGB1a are located in close proximity in rod OS. Overall, our results are consistent with the model shown in Figure 6. The novel aspect of this model is that peripherin-2 by binding to both, rhodopsin and the CNG channel, physically couples the most proximal protein of the light transduction cascade (rhodopsin) with the most distal protein (the CNG channel) in a narrow spatial microcompartment encompassing the disk rims and the neighboring plasma membrane. One could imagine two principal scenarios where the supramolecular organization of these proteins might be relevant. First, the complex could play a structural role in the formation and maintenance of rod OS structure. Mutations in peripherin-2 as well as in rhodopsin are associated with impaired disk morphogenesis and stability resulting in shortened and deteriorated rod OS (15–18). Structural impairments of OS are also seen upon mutation of ROM1, another photoreceptor-specific protein that binds to peripherin-2 (19), and in CNGB1a-knockout mice lacking the peripherin-2-binding GARP domain (20). Taken together, these findings imply that the integrity of the rhodopsin/peripherin-2/ROM1/CNG channel complex is crucial for morphogenesis and long-term stability of rod OS. Loss or functional impairment of any of the constituents of the complex will thus lead to more or less severe structural defects. In support to a structural role of the rhodopsin/peripherin-2 interaction, the addition of rhodopsin to the reconstituted peripherin-2/ROM1 complex in lipid vesicles was recently reported to induce the formation of disk rim-like structures in vitro (21).

The second scenario refers to a functional role of the complex in visual transduction. By binding to both, rhodopsin and the CNG channel, peripherin-2 helps to generate a microcompartment in
the rim disk region that could have evolved to optimize the sensitivity and precision of light transduction. Recently, it was reported that a substantial portion of the rod phosphodiesterase (PDE6) is located at the disk rims of rod OS (22). GARP2 that was shown to bind PDE6, peripherin-2, and the CNG channel subunits (3,23,24) could serve as an adaptor protein that anchors PDE6 to the disk rim. Another study also reported a physical interaction of PDE6 and the rod CNG channel (25). The proposed complex would be exquisitely efficient because the distance between the light harvesting rhodopsin, the guanosine monophosphate (cGMP)-hydrolyzing PDE6 and the channel that translates changes in cGMP into changes of the Na+/Ca2+ flux would be extremely short. In contrast to rhodopsin, the CNG channel and peripherin-2 were reported to be absent in the central disk region (2,26,27). Consequently, in this region, rhodopsin is physically uncoupled from the CNG channel. At the moment, only one can speculate about the exact functional role of the differential microcompartmentalization of rhodopsin in the central and peripheral part of the disks. Owing to the shorter diffusion distance for cGMP, however, the disk rim-associated complex is expected to operate at lower light intensities and faster kinetics. Such an optimization could be important in rods, which are tailored to detect extremely low light levels. In contrast, in the less light-sensitive cones, coupling of the light sensor to the channel seems less important. In agreement with this hypothesis, the cone CNG channel subunits lack the GARP domain that is required for the interaction with peripherin-2 (3,28,29).

**MATERIALS AND METHODS**

**Animals**

All procedures concerning animals were performed with permission of local authorities (Regierung von Oberbayern).

**Molecular biology**

Full-length CNGB1a, GARP2, peripherin-2 and rhodopsin were PCR-amplified from mouse retinal cDNA and subcloned into the pcDNA3.1Myc/His expression vector (Invitrogen). For FRET measurements and for *in vitro* co-IP studies, the FRET-optimized YFP and CFP derivatives citrine or cerulean (30–32) were introduced N- or C-terminally to the respective constructs by standard cloning procedures. Peripherin-2 deletion mutations were obtained by site-directed mutagenesis (QuikChange Lightning Mutagenesis Kit, Agilent Technologies) and overlap PCR techniques. All constructs amplified by PCR were completely sequenced prior to use. For the FRET studies on rod OS, the appropriate FRET constructs were subcloned to the previously described single-stranded pAAV2.1 vector (33) containing a human rhodopsin (hRHO) promoter.

**Retina preparation and protein biochemistry**

For *in vivo* co-IPs and for quantitative mass spectrometry, freshly dissected retinas were homogenized in 1% Triton X-100 solution under normal daylight illumination, which
results in bleached rhodopsin. Subsequently, cell debris was removed by 5000 × g centrifugation for 15 min, and protein concentration of the supernatant was determined by Bradford. For mass spectrometry, 50 μl of the magnetic beads (Dynabeads® Protein A, Invitrogen) were pre-incubated with 5 μg of the CNGB1 antibody (34) in a total volume of 500 μl of PBS for 2 h at 4°C. After the removal of PBS, the pre-coupled beads were incubated overnight at 4°C with 500 μg of total retinal protein per single reaction. Next steps were performed according to manufacturer’s instructions. After the removal of magnetic beads, the immunoaffinity purified proteins were analyzed via LC MS/MS as described previously (35).

For in vivo co-IP experiments, anti-B1a, anti-rhodopsin [mouse anti-Rho 1D4, Thermo Scientific) and anti-peripherin-2 (mouse anti-Prph2 2B7 (36)] antibodies were used followed by the same protocol as described earlier for the mass spectrometry. The anti-B1a antibody recognizes the distal C-terminus of the rod-specific CNGB1a and the olfactory-specific CNGB1b subunit (34). Immunopurified proteins were separated on a 6–12% SDS–PAGE gradient gel and proceeded for western blotting. Antibodies were used in the following dilutions: rabbit anti-B1a, 1 : 1000; mouse anti-Prph2, 1 : 1000; mouse anti-Rho, 1 : 2000; mouse anti-myc, 1 : 2000 (Cell Signaling); mouse anti-GFP (Clontech), 1 : 2000. In vitro co-IPs were performed as described previously (23). Rod opsin signals from untreated HEK293 protein lysates are detected as a smear on western blot owing to an extensive rod opsin glycosylation. Hence, prior to the sample loading to the SDS–PAGE gel, a deglycosylation step was included using PNGase F (New England Biolabs) according to the instructions of the manufacturer. For in vitro co-IPs with the anti-myc or anti-GFP antibody, the μMACS GFP or myc-tagged beads were used (Miltenyi Biotec). All steps were performed in accordance with the manufacturer’s protocol.

Photometric FRET measurements

Digital FRET measurements were performed as described (37). For confocal FRET measurements, HEK293 cells were grown in 5-cm cell culture dishes (Greiner bio-one) and transiently transfected using the calcium phosphate method. After 24–48 h, the cells were washed and maintained in buffer solution composed of 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose, 10 mM Na-HEPES, pH 7.4, at room temperature.
Cells were imaged using a Leica TCS SP8 confocal microscope with a 20× water objective (1.0 numerical aperture). Excitation of cerulean was performed with a 448-nm laser followed by emission measurements between 483±16 nm. Citrine was excited with a 514-nm laser beam, and emission was measured between 542±17 nm. FRET images were obtained by 448-nm excitation and 542±17-nm emission. Cerulean, FRET and citrine images were acquired simultaneously, and all images were performed under the same laser intensity, photomultiplier gain and pinhole settings. The intensities of the signals were subtracted from the background, and FRs were calculated according to the three cube FRET equations described in (37). Cell membrane was defined as region of interest, and images of cells with over- or under-saturated cerulean or citrine signals were omitted from further analysis. For the conversion of fluorescence intensities obtained from measurements in cells expressing separate cerulean- and citrine-labeled proteins into molar cer/citr ratios, we used a correction factor, $R_\text{c} = 3$ (37). To calculate $FR_{\text{max}}$ and to obtain the binding curves, FRs of the respective FRET pairs were plotted versus the cer/citr ratios and fitted to a hyperbolic function: $(P_1 \times X)/(P_2 + X)$. $X$ is the FRET values of the single cells and P1 and P2 were calculated by 100 iterations for each FRET pair. $P_1$ is the limiting value of the function and equals $FR_{\text{max}}$. For the determination of the relative binding affinity, the following equation was used: $(FR_{\text{maxPrph2}}/FR_{\text{maxrOps}}) \times 100$.

**Preparation of OSs**

Single retinas were dissected and transferred to 200 μl of PBS. The OSs were mechanically separated by mixing the solution for 10–20 s on a standard shaking device. The solution was spun for few seconds to remove cell debris. The supernatant containing the OSs was transferred to cell culture dishes (ibiTreat, ibidi, Martinsried, Germany). Prior to the FRET measurements, the OSs were allowed to sediment for ~15 min.

**rAAV preparation and subretinal injections**

The production of single-strand AAVs and the procedure of subretinal injections were described previously (33). A total of $10^8-10^{10}$ rAAV particles were delivered by a single injection.

**Immunoelectron microscopy**

The preparation for immunoelectron microscopy was performed as previously described (38). Labeled LRWhite ultrathin sections were analyzed in a transmission electron microscope (Tecnai 12 BioTwin; FEI, Eindhoven, The Netherlands). Images were obtained with a charge-coupled device camera (SIS Megaview3; Surface Imaging Systems), acquired by analySIS (Soft Imaging System) and processed with Adobe Photoshop CS. Antibodies used were as follows: anti-B1a, anti-Rho and anti-Prph2 (PER5H2) (39).

**Statistics**

All values are given as mean ± SE, and $n$ is the number of animals or trials. Unless stated otherwise, an unpaired student’s $t$-test was performed for the comparison between two groups. Statistical significance is given as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. If multiple comparisons were made, significance was tested by analysis of variance followed by Dunnett’s test. For analysis of the LC MS/MS data, one-way ANOVA was used.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**ACKNOWLEDGEMENTS**

We thank Berit Noack for excellent technical support. We also thank Muna Naash and Robert Molday for the gift of the peripherin-2 antibody.
Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft; the FAUN-Stiftung, Nurnberg (to U.W.); the European Community FP7/2009/241955 (SYSCILIA) (to U.W.); and the Bundesministerium für Bildung und Forschung, (HOPE2) (grant number 0314106 to U.W.).

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