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Photoreceptor Sensory Cilia and Inherited Retinal Degeneration

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Abstract

The outer segments of photoreceptor cells are specialized sensory cilia, and share many features with other primary and sensory cilia. Like other cilia, photoreceptor sensory cilium (PSC) comprises a membrane domain of outer segment and its cytoskeleton. We have recently identified the protein components of mouse PSCs, and found that the list of PSC proteins, called the PSC proteome, contains many novel cilia proteins. Studies have shown that many of the identified retinal degeneration (IRD) disease genes encode proteins which are part of the PSC. Furthermore, mutations in genes encoding proteins expressed both in photoreceptors and other cilia result in systemic diseases, such as Usher syndrome, Bardet-Biedl syndrome (BBS), and Senior-Loken syndrome that involve retinal degeneration along with other disorders consequent to cilia dysfunction such as deafness and polycystic kidney disease. Based on these findings, we hypothesize that genes that encode proteins required for formation of PSCs are good candidate retinal degeneration disease genes. This chapter will summarize our studies on identifying novel PSC proteins from the PSC proteome. As an example of these studies, we demonstrated that tetratricopeptide repeat domain 21B (TTC21B) protein is a novel PSC protein and is required for normal cilia formation in primary and photoreceptor sensory cilia.

26.1 PSC Proteins Involved in Inherited Retinal Degenerations

Inherited retinal degenerations (IRDs) are characterized by progressive dysfunction and death of photoreceptor cells, and are genetically heterogeneous, with over 140 disease genes identified to date (RetNet 2008). IRDs occur in non-syndromic and syndromic forms. It is estimated that 65% of retinitis pigmentosa (RP) cases are non-syndromic, and 30–35% of individuals with RP have associated a broad set of non-ocular disease, including Alstrom, Bardet-Biedl, Joubert, Meckel, Senior Loken/nephronophthisis and Usher syndromes (Hartong et al. 2006; Badano et al. 2006; Daiger et al. 2007; Hildebrandt and Zhou 2007). Recent evidence indicates that dysfunction of sensory cilia is the underlying cause of these multisystemic disorders (Badano et al. 2006). This highlights the importance of recognition of photoreceptor outer segments as specialized sensory cilia, and also explains the connection of IRDs at a mechanistic level to a larger class of systemic cilia disorders, in which retinal degeneration is found in association with multiple cilia-related diseases, including cystic renal disease, polydactyly, mental retardation, obesity and gonadal malformations, diabetes, and situs inversus (Badano et al. 2006). The inclusion of IRDs in the larger class of cilia disorders allows information from investigations of other types of cilia to be applied to PSCs and inherited retinal disorders. The synergy also operates in reverse, so that findings from studies of photoreceptor biology have the potential to be relevant to understanding cilia function and diseases in general.

While significant progress has been made in identifying IRD disease genes, the genes which harbor mutations that cause disease in approximately 50% of IRD patients remain to be identified (Hartong et al. 2006; Badano et al. 2006; Daiger et al. 2007; Hildebrandt and Zhou 2007). Finding the genetic cause of IRDs has become increasingly important, as the recent reports of early successes with gene therapy for LCA2 indicate that we are entering an era of genetic therapies for IRDs (Maguire et al. 2008). Notably, the majority of the IRD disease genes identified over the past 3 years encode proteins that were first identified to be part of photoreceptor sensory cilia (PSCs) in the PSC complex proteome (see below) (Chang et al. 2006; Valente et al. 2006; Sayer et al. 2006; den Hollander et al. 2006). Based on these observations, we propose that novel cilia proteins detected in the PSC proteome are important for PSC structure and function, and genes that encode these novel cilia proteins are thus good candidate disease genes for IRDs. As described below, we have been testing these hypotheses by identifying novel PSC proteins that are required for correct cilia formation and/or function, and then screening the genes that encode functionally validated PSC proteins for mutations that cause IRDs and related ciliopathies.

26.2 Structure of Photoreceptor Sensory Cilium Complex

Photoreceptor outer segments are specialized sensory cilia. This is not a new idea, but rather a new appreciation of a concept that has been in the literature for many years (De Robertis 1956; Allen 1965; Matsusaka 1974). Until recently, the importance of primary and sensory cilia in biology and disease is being more broadly recognized (Singla and Reiter 2006; Christensen et al. 2007; Slough et al. 2008; Breunig et al. 2008; Simons and Mlodzik 2008). Primary cilia are present on most vertebrate cell types. These structures are typically sensory organelles, and are involved in many critical aspects of cell biology. For example, sensation of flow by primary cilia is required for maintenance of renal nephron structure and body axis determination. Primary cilia play important roles in various aspects of development, such as planar cell polarity as regulated by hedgehog and wnt signaling (Singla and Reiter 2006; Davis et al. 2006; Gerdes et al. 2007).

The sensory cilia elaborated rod and cone photoreceptors are among the largest of mammalian cilia (Pan et al. 2005; Yang et al. 2005). Like other cilia, photoreceptor sensory cilium comprises a membrane domain of outer segment and its cytoskeleton. The membrane domain of outer segments is highly specialized, in this case for light detection, with the proteins required for phototransduction located in or associated with the membrane discs stacked in tight order at ~30 per micron along the axoneme. The cytoskeleton of PSCs includes a basal body, transition zone (also called the 'connecting cilium'), axoneme and rootlet. The axoneme begins at the basal body, passes through a transition zone and into the outer segment. The basal bodies also nucleate the ciliary rootlet, which extends into the inner segment (Yang et al. 2005). The transition zone in photoreceptor sensory cilia is analogous to transition zones in other cilia, and is the region where the triplet microtubule structure of the basal bodies converts to the doublet microtubule structure of the axoneme (Horst et al. 1990). This region was first called the 'connecting cilium' by DeRobertis in 1956, when he was studying some of the first electron micrographs of photoreceptor cells (De Robertis 1956). This name was applied before the structure of cilia was completely defined, and it was understood that the axoneme of photoreceptor outer segments extends through the transition zone, and for up to 2/3rds of the length of the outer segment (De Robertis 1956; Kaplan et al. 1987). In recognition of the homology between PSCs and other cilia, this region will be called the transition zone below

26.3 Protein Components of Photoreceptor Sensory Cilium: PSC Proteome

The recognition of photoreceptor outer segments as cilia allows for consideration of the whole photoreceptor sensory cilium as a biologic structure, which is valuable for the study of

photoreceptor cell biology and disease pathogenesis. A number of proteins have been identified to be components of the cytoskeletons of PSCs, primarily through study of proteins produced by retinal degeneration disease genes. These include BBS2, BBS4, BBS7, BBS8, CEP290, CIP98(DFNB31), GPR98, IQCB1, LCA5, MYO7A, Nephrocystin-1 (NPHP1), Nephroretinin (NPHP4), PCDH15, RP1, RPGR, RPGRIP1, TTC8, USH1G, Usherin (USH2A) (Liu et al. 1997; Hong et al. 2000; Liu et al. 2002; Zhao et al. 2003; Ansley et al. 2003; Liu et al. 2004; Otto et al. 2005; Roepman et al. 2005; Reiners et al. 2005a; Reiners et al. 2005b; Chang et al. 2006; Fliegauf et al. 2006; Liu et al. 2007; den Hollander et al. 2007; Maerker et al. 2008). This list presents only a small portion of PSC cytoskeleton proteins. To initiate studies of PSCs from a broader perspective, we reported a complete proteome of mouse PSCs (Liu et al. 2007).

The PSC proteome identified by ≥ 3 unique peptides contains 1968 proteins, including ~1,500 proteins not detected in cilia from lower organisms. This includes 105 hypothetical proteins, and many cilia proteins not previously identified in photoreceptors. Several measures show that the proteome is highly accurate, and includes the majority of proteins (95%) in PSCs. Analyses of PSC complexes from rootletin knockout mice, which lack ciliary rootlets and separate from the inner segments of photoreceptor cells easily without the major inner segment component of the PSC complex cytoskeleton, confirm that 1,185 of the identified PSC complex proteins are derived from the outer segment (Yang et al. 2005; Liu et al. 2007). The PSC complex proteome accelerates greatly the progress toward improved understanding of how photoreceptor cilia are built and maintained, and how these processes are disrupted in disease.

26.4 Novel Photoreceptor Cilia Proteins in PSC Proteome

The PSC complex proteome contains many cilia proteins not previously identified in photoreceptors, including 13 proteins produced by genes which harbor mutations that cause cilia diseases. We have selected a subset of ~200 novel proteins from the PSC complex proteome for initial evaluation. These novel proteins were selected based on having features that are shared with known PSC and other cilia proteins. This includes: 1. proteins with WD repeat, and tetratricopeptide (TPR) domains, which are common in IFT proteins (Jekely and Arendt 2006); 2. proteins with other domains shared with IRD disease proteins, such as coiled-coil and GTP-binding domains (Fan et al. 2004; Cantagrel et al. 2008); 3. hypothetical proteins that are especially abundant in the PSC based on the mass spectrometry data; and 4. proteins that are shared with the Ciliaproteome, a meta-analysis of cilia datasets from other organisms (Gherman et al. 2006). In this section, we will describe briefly the approaches that have been used to study the novel PSC proteins in our laboratory.

26.4.1 Subcellular Locations of Candidate Novel PSC Proteins

The proteins selected were either hypothetical or not previously identified in cilia. In hopes of gaining insights into the function of these proteins, we first determined their location in PSCs or renal cilia by expressing epitope-tagged versions of the proteins from a pCAG-V5-cDNA-IRES-EGFP Gateway expression vector. The cDNA clones were obtained from the Invitrogen Ultimate ORF clone collection, or the MGC and IMAGE cDNA clone collections, or amplified by RT-PCR from mouse retinal cDNA. The V5-tagged PSC cDNA plasmids were expressed in a ciliated mouse inner medullary collecting duct (mIMCD3) cell line, which stably expresses somatostatin receptor 3 (Sstr3)-EGFP in the primary cilia (Berbari et al. 2008), as well as in the photoreceptor cells of neonatal rats by using in vivo electroporation technique (Matsuda and Cepko 2004). The location of the V5-tagged proteins were assessed by immunostaining with anti-V5 antibodies in the mIMCD3 cells 48 h after transfection or in the photoreceptor cells 4 weeks following transfection via in vivo electroporation.

Observations from our initial studies showed that a clear V5 signal was found for the majority of the novel proteins evaluated to date. This includes 15 proteins specifically in PSC cytoskeletons, 13 proteins in PSCs, 32 proteins in the PSC plus other parts of the cell, and 26 proteins in the inner segment. Four proteins localized to the basal bodies and/or cilia of the mIMCD3 cells. The reliability of the location of the recombinant proteins were confirmed by anti-peptide antibodies we generated for 4 of the novel PSC proteins. The 28 proteins novel identified to be part of PSCs and their cytoskeleton are of particular interest, and are being studied further in the functional analyses as described below.

26.4.2 Functional Analysis of Novel PSC Proteins in Photoreceptor and Renal Cilia

26.4.2.1 shRNAs Against Novel PSC Genes—To investigate the function of validated novel PSC in cilia, shRNA-mediated knockdown techniques were used in renal cells in culture and photoreceptor cells in vivo. Three to four oligonucleotides encoding the identified shRNA sequences were cloned into the pCAG-mir30-puro vector. The activity of the cloned and sequence verified shRNAs were quantified following transfection into mIMCD3 cells using quantitative RT-PCR assays with TaqMan probes (Giulietti et al. 2001). For genes that are not expressed in mIMCD3 cells, the shRNA plasmids were co-transfected with pCAG-V5-*cDNA*-IRES-EGFP plasmids containing the cDNAs of interest, and the loss of the EGFP signal taken as evidence of successful knockdown (Matsuda and Cepko 2004). shRNA sequences that are verified to provide significant knockdown of the target gene ($\geq 70\%$) were used for the phenotypic analyses described below. Non-targeted shRNAs (shRNA-NT) and shRNAs for luciferase (shRNA-Luc) were used as controls for these and all other shRNA experiments.

26.4.2.2 Evaluation of Phenotypes of shRNA Knockdown in mIMCD3 Cells and PSCs—We used Sstr3-EGFP mIMCD3 cells to assess the function of the novel PSC proteins in renal primary cilia. Cells transfected with the validated pCAG-mir30-puro-shRNA plasmids were subjected to selection with puromycin for 72 h, and then serum starved for 24 h to stimulate cilia formation (Tucker et al. 1979). The cells were labeled with acetylated α -tubulin antibody to mark axonemes, and then were scored for the presence and length of the axonemes and cilia, as indicated by the acetylated α -tubulin signal and Sstr3-EGFP signal, respectively.

To assess the function of novel PSC proteins in photoreceptor cilia, we used in vivo electroporation to transfect rat photoreceptor cells with a combination of two plasmids: the pCAG-mir30-puro-shRNA plasmid with a validated shRNA against the PSC gene of interest and the pCAG-Flag-Prph2-IRES-EGFP plasmid. The IRES-EGFP marks the transfected cells, and the Flag-Prph2 allows for evaluation of their outer segment structure. Vibratome sections with EGFP signal and good morphology were stained with anti-Flag antibodies followed by three-dimensional volume reconstructions of the confocal images using Volocity 3D imaging software (Improvision, Waltham, MA). The structures of the outer segments of transfected photoreceptor cells will be compared with those of the control shRNA-NT transfected retinas.

26.5 TTC21B Protein in Photoreceptor Sensory Cilia and Renal Primary Cilia

So far, we have identified dozens of novel cilia proteins using the approaches described above. In collaboration with other investigators, we are screening several of the validated PSC genes for mutations in patients with IRDs and related ciliopathies. As one example of these studies, we have identified TTC21B as a novel cilia protein. Functional studies showed that Ttc21b was required for normal PSC structure and renal primary cilia formation. *TTC21B* was initially selected for analyses for several reasons. TTC21B protein contains TPR domains, which are common to IFT proteins; Ttc21b was relatively abundant in the PSC complex proteome (14 unique peptides); and Ttc21b was shared with the Ciliaproteome dataset (Jekely and Arendt 2006; Gherman et al. 2006; Liu et al. 2007). Other investigators have recently reported that TTC21B is a retrograde IFT protein (Tran et al. 2008).

26.5.1 TTC21B Localizes to the Basal Bodies and Transition Zone of Primary and Photoreceptor Sensory Cilia

To identify the location of the TTC21B protein in renal and photoreceptor cilia, we expressed V5-tagged human TTC21B in renal IMCD3 cells and photoreceptor cells, and developed antibodies against mouse *Ttc21b*. Staining of the transfected mIMCD3 cells with anti-V5 antibodies shows that TTC21B is located at the base of the primary cilia (Fig. 26.1a). In addition, there is a portion of the TTC21B signal that extends beyond the basal body. Results from the transfected photoreceptor cells shows that the V5 tagged TTC21B protein is mainly located at the transition zones of PSCs, with a small portion of signal extending into the axonemes (Fig. 26.1b). This result was confirmed by anti-*Ttc21b* antibody staining in mouse retina. As shown in Fig. 26.1c, *Ttc21b* is located in the transition zones of PSCs, proximal to the axonemes which were labeled by anti-Rp1h antibody.

26.5.2 TTC21B is Required for Primary Cilia and Photoreceptor Sensory Cilia Formation

To determine if *Ttc21b* is required for cilia formation, we generated two shRNAs against *Ttc21b*, and cloned them into the pCAG-mir30-puro vector. Both shRNAs provide 70–80% knockdown of *Ttc21b* mRNA levels following transfection into mIMCD3 cells. We then evaluated the effects of *Ttc21b* shRNA-mediated knockdown on the structures of primary cilia and PSCs. In cultured mIMCD3 cells, transfection of either of the shRNA-*Ttc21b* plasmids resulted in notable shortening of primary cilia ($1.6 \pm 1.1 \mu\text{m}$), as demonstrated by shorter axonemes and lack of the Sstr3-EGFP signal. In contrast, cilia in cells transfected with a control non-targeted shRNA (shRNA-NT) are long ($6.4 \pm 1.3 \mu\text{m}$). Co-transfection of shRNA-resistant human *TTC21B* cDNA with the shRNA-*Ttc21b* restored cilia to almost full length ($4.8 \pm 1.1 \mu\text{m}$; $p < 0.0001$) (data not shown). These data suggest that TTC21B is required for formation of normal primary cilia.

In a similar fashion, we also used the in vivo electroporation technique to assess the effect of shRNA-mediated knockdown of novel PSC proteins on photoreceptor outer segment structure. The 3D volume reconstructions of the confocal images demonstrate that *Ttc21b* knockdown results in abnormal PSC structure, with ‘bulbs’ in place of or at the distal end of the outer segments of most transfected photoreceptor cells. In contrast, the outer segments of control shRNA-NT transfected photoreceptors are slender rods (data not shown). A recent publication suggests that TTC21B is a retrograde IFT protein, and demonstrated similar bulbs at the distal ends of renal cilia following *Ttc21b* knockdown, which are thought to be caused by loss of the retrograde IFT activity (Tran et al. 2008).

26.6 Future Direction: Screening Novel PSC Genes for Mutations that Cause IRDs

As mentioned above, many IRDs and related ciliopathies are caused by mutations in genes that encode components of PSCs and other cilia (Badano et al. 2006; Liu et al. 2007; Hildebrandt and Zhou 2007). While significant progress has been made in identifying IRD disease genes, the genes which harbor mutations that cause disease in approximately half of IRD patients remain to be identified (Hartong et al. 2006; Badano et al. 2006; Daiger et al. 2007; Hildebrandt and Zhou 2007). To identify the candidate disease genes for IRDs, we have initiated the screening for mutations in validated PSC genes in patients with cilia related disorders, including recessive and dominant RP, LCA, and other syndromic cilia disorders such as BBS. This work of mutation screening is being performed in collaboration with several other investigators. For future studies, we will continue testing the hypothesis that genes that encode novel PSC proteins are good candidate disease genes for IRDs and related ciliopathies. We are optimistic that continued screening of validated PSC genes for mutations in patients with ciliopathies will lead to the identification of additional disease genes.

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Fig. 26.1.

Ttc21b in mIMCD3 cells and retina. **a.** V5-tagged TTC21b (*red, arrow*) is located at the base of cilia in transfected mIMCD3 that stably express the cilium marker Sstr3-EGFP (*green*). **b.** V5-tagged TTC21b (*red*) is located at the transition zones in transfected photoreceptor cells with pCAG-V5-TTC21B-IRES-EGFP, with a small portion of signal extending into the axonemes. **c.** Antibodies to mouse Ttc21b (*red, arrow*) show that the protein is located in the transition zone of photoreceptor cilia. The axonemes of the PSCs are shown by antibodies to Rp1h (*green*). *Blue*, nuclei; Ax, axoneme; IS, inner segment; ONL, outer nuclear layer