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Abstract

Primary cilia are microtubule-based sensory organelles that are involved in the organization of numerous key signals during development and in differentiated tissue homeostasis. The formation and resorption of cilia highly depend on the cell cycle phase in replicative cells, and the ubiquitin proteasome pathway (UPS) proteins, such as E3 ligases and deubiquitinating enzymes, promote microtubule assembly and disassembly by regulating the degradation/availability of ciliary regulatory proteins. Also, many differentiated tissues display cilia, and mutations in genes encoding ciliary proteins are associated with several human pathologies, named ciliopathies, which are multi-organ rare diseases. The retina is one of the organs most affected by ciliary genes mutations because photoreceptors are ciliated cells. In fact, photoreception and phototransduction occur in the outer segment, a highly specialized neurosensory cilium. In this review, we focus on the function of UPS

	proteins in ciliogenesis and cilia length control in replicative cells and compare it with the scanty data on the identified UPS genes that cause syndromic and non-syndromic inherited retinal disorders. Clearly, further work using animal models and gene-edited mutants of ciliary genes in cells and organoids will widen the landscape of UPS involvement in ciliogenesis and cilia homeostasis.
Keywords (separated by '-')	Cilia - Ciliogenesis - Ciliopathy - DUBs - Ubiquitin-proteasome system - Photoreceptor

By the Tips of Your Cilia: Ciliogenesis in the Retina and the Ubiquitin-Proteasome System

13

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Abstract

Primary cilia are microtubule-based sensory organelles that are involved in the organization of numerous key signals during development and in differentiated tissue homeostasis. The formation and resorption of cilia highly depend on the cell cycle phase in replicative cells, and the ubiquitin proteasome pathway (UPS) proteins, such as E3 ligases and deubiquitinating enzymes, promote microtubule assembly and disassembly by regulating the degradation/availability of ciliary regulatory proteins. Also, many differentiated tissues display cilia, and mutations in genes encoding ciliary proteins are associated with several human pathologies, named ciliopathies, which are multi-organ rare diseases. The retina is one of the organs most affected by ciliary genes mutations because photoreceptors are ciliated cells. In fact, photoreception and phototransduction occur in the outer segment, a highly specialized neurosensory cilium. In this review, we focus on the function of UPS proteins in ciliogenesis and cilia length control in replicative cells and compare it with the scanty data on the identified UPS genes that cause syndromic and non-syndromic inherited retinal disorders. Clearly, further work using animal models and gene-edited mutants of ciliary genes in cells and organoids will widen the landscape of UPS involvement in ciliogenesis and cilia homeostasis.

Keywords

Cilia · Ciliogenesis · Ciliopathy · DUBs · Ubiquitin-proteasome system · Photoreceptor

13.1 The Retina and Photoreceptors

The retina is a multilayer neurosensory tissue that covers the inner surface of the eye. In vertebrates, the retina is formed by at least six different highly specialized neuronal cell types [1]. The light-sensitive photoreceptor cells are responsible for absorption of the light stimuli (capturing photons) and the initiation of the phototransduction cascade, which eventually transmits the electric signal to the visual centers in the brain [2]. There are two types of photoreceptors, rods and cones. Cones are responsible for visual acuity and color

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perception in photopic conditions, while rods are sensitive in dim light and are responsible for scotopic and non-color vision [3].

The proteins that are implicated in photoreception and phototransduction are localized in a specialized photoreceptor compartment, the outer segment (OS), which is a highly specialized sensory cilium that contains ordered stacks of membranous disks. However, the OS lacks the protein synthesis machinery and, thus, all the components of the OS are synthesized in the inner segment (IS) of photoreceptors and transported to the OS through a microtubule ciliary gate, known as the connecting cilium [4]. The tips of photoreceptor OS are physically in contact with the retinal pigment epithelium (RPE), a single layer of pigmented cells, which participates in maintaining the visual cycle [5] as well as in the daily shedding of OS disks by phagocytosis of the photoreceptor tips [6]. Dysregulation by either genetic mutations or external factors of such a regulated morphological and functional organization triggers apoptosis of photoreceptor and related neurons, thus leading to retinal dystrophy and blindness [7].

13.2 Photoreceptor Cilia

Primary cilia are microtubule-based extensions of the apical plasma membrane that act as cell sensors of external cues. Cilia are signaling receptor hubs that modulate developmental signaling, such as sonic hedgehog, Wnt, and platelet-derived growth factor pathways. Cilia are also receptors of external stimuli and transducers of sensory perception and are involved in chemosensation, olfaction, mechanosensation, and photoreception [4]. It is well known that many ciliary proteins are associated with several human rare diseases, named ciliopathies, which include cystic kidney disease and retinal degeneration traits, such as Bardet–Biedl syndrome (BBS), Usher syndrome, Joubert syndrome, Senior-Locken syndrome, or Meckel–Gruber syndrome, among others [8, 9]. Ciliopathies are usually syndromic disorders since many different tissues and organs display ciliated cells, among them the retina, cochlea, and kidney.

The cilium is composed of a long microtubule doublet, called axoneme, surrounded by an external membrane that is continuous with the plasma membrane of the cell. The axoneme is grown directly from the distal end of a mother centriole (or basal body) through a multistep process, named ciliogenesis or cilia formation, which requires microtubule polymerization and intraflagellar transport (IFT) for cilium elongation [10]. IFT is a bidirectional transport of cargo proteins from the base of the cilium to the tip (anterograde transport) and vice versa, from the tip to the base of the cilium (retrograde transport). Different molecular motors facilitate trafficking: for instance, kinesin-II and cytoplasmic dynein 2, respectively, involved in anterograde and retrograde transport, associate with specific IFT proteins which are organized into two major complexes for cargo transport. The IFT-B complex is involved in anterograde trafficking whereas IFT-A is predominantly involved in retrograde trafficking [11].

In photoreceptors, the microtubule region that connects the IS, where all the proteins are synthesized, with the membranous disks of the OS, that is, the region between the basal body and the base of the cilium, receives several names, i.e., transition zone, ciliary gate, or connecting cilium. The transition zone (where the axoneme transitions from the triplet to the doublet microtubule conformation) serves at least two functions, as a docking module of the cilium to the membrane as well as a regulated and restrictive gate through which the cargo proteins are transported to and from the OS using the IFT machinery [4].

13.3 DUBs and the Ubiquitin-Proteasome System (UPS)

The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin-proteasome system. Ubiquitination is a post-translational modification that consists in the attachment of ubiquitin (Ub) to a protein substrate [12]. For many proteins, the attachment of ubiquitin and growth of polyubiquitin chains is

an obligatory step in their degradation [13]. Ubiquitination is a highly dynamic and reversible reaction where ubiquitin is conjugated by the serial action of specific ubiquitin ligases and cleaved off from substrates by deconjugating proteases, also named deubiquitinating enzymes (DUBs) [13]. Ubiquitin conjugation relies on a hierarchically consecutive activity of E1, E2, and E3 ligases. By far, the largest family is that of E3 ligases, which are subclassified into three main groups according to their mode of ubiquitin ligation [14]. Concerning DUBs, most are cysteine proteases, but according to their structure and catalytic motifs they are also subclassified into six different families [15]. The human genome contains more than 600 hundred E3 ligases and close to 100 DUBs. The world of post-translational peptide conjugation has also expanded to include other ubiquitin-like peptides (e.g., SUMO, NEDD8, ISG15, and Atg5) [16], all of which are molecular tags that regulate protein fate.

Post-translational ubiquitin and ubiquitin-like modifications play an important role during photoreceptor differentiation and ciliogenesis. For instance, the key transcription factor for the determination of the photoreceptor cell fate, Nr2e3, is post-translationally modified via SUMOylation by Pias3, turning it into both a potent repressor of cone-specific gene expression and an activator of rod-specific genes in rods [17]. Moreover, previous studies have analyzed the expression levels and drawn the expression map of genes related to SUMO and Ub pathways in the mouse retina, thus indicating that some of them could be possible regulators of photoreceptor differentiation and/or candidate genes for causing retinal dystrophies (e.g., *Cbx4*, *Tls*, *Hdac4*, *Uchl-1*, *Atxn3*, *Usp45*, *Usp53*, *Usp54*) [18, 19].

13.4 UPS and Ciliogenesis in Replicative Cells

In vertebrates, ciliogenesis and cell division are mutually exclusive because the centrioles must be released from the plasma membrane to function in the mitotic apparatus. Therefore, in replicative cells, ciliogenesis and cilia resorption are highly regulated processes that depend on the cell cycle phase, the microtubule network organization, cellular proteostasis, and cilia-mediated signaling cues. On the other hand, many cells produce cilia after escaping cell cycle and entering into differentiation. The proteins and signals that regulate ciliogenesis and cilium disassembly in replicative cells might be common to those involved in cilia maintenance in quiescent and differentiated cells, such as photoreceptors, although some partners might be cell type- or organ- specific. Since microtubule polymerization and depolymerization are highly dynamic, any alteration in the equilibrium of these two processes will directly affect cilium formation or resorption. Although the mechanistic details are not yet fully understood, post-translational modifications of centrosomal and ciliary proteins are key to ciliogenesis [20].

Previous studies have identified UPS proteins that localized at the cilium and might regulate cilium formation, for instance, the chaperone VCP (valosin-containing protein) (involved in ubiquitin signaling quality control and positive regulator of misfolded protein degradation), the ubiquitin-activating enzymes UBA1 and UBA6, and the E3 ubiquitin ligases NEDD4L (neural precursor cell-expressed, developmentally downregulated 4-like) and MYCBP2 (MYC-binding protein 2) [21]. Other studies have identified UPS factors as positive or negative regulators of ciliogenesis and cilium length [22, 23]. Indeed, instrumental UPS proteins involved in cell cycle regulation, such as the anaphase-promoting complex (APC), are also regulating cilia assembly/disassembly. APC is recruited to basal bodies in quiescent cells where it promotes the degradation of KIF2A (a microtubule depolymerase), but different subunits of APC may serve different regulatory functions concerning the cilia, and APC's role is most probably that of a modulator of ciliary microtubule depolymerization depending on the cell phase and requirements [24].

For instance, CUL3 is an E3 ubiquitin ligase that participates in the ubiquitination of many proteins. Interaction of CUL3 with one of its

substrate adaptors, KCTD17, is required to polyubiquitinate trichoplein, a negative regulator of ciliogenesis, and remove it from mother centrioles. Therefore, CUL3-KCTD17 is a positive regulator of ciliogenesis, since it targets trichoplein, thereby inactivating Aurora A and allowing axoneme extension [25]. Similarly, VHL (von Hippel-Lindau) is a tumor suppressor that enhances primary cilia formation. VHL inactivation induces Aurora kinase A activity, thus causing regression of the primary cilium by promoting histone deacetylase-dependent tubulin depolymerization of the ciliary axoneme [26]. Another example is the E3 ubiquitin ligase MIB1, a component of centriolar satellites that acts as a negative regulator of ciliogenesis by ubiquitinating key ciliogenesis-promoting factors, targeting them for degradation and, as a consequence, suppressing primary cilium formation [23]. Furthermore, another E3 ligase important for the correct cilia formation is UBR5, which ubiquitinates CSPP, a centrosomal protein essential for ciliogenesis [27]. In addition, two other E3 ligases, BBS11/TRIM32 and MARCH7, are localized in the centrosome and ubiquitinate ephrocystin-5 (NPHP5), protein involved in the control of ciliogenesis [28].

Concerning DUBs, depletion of USP21 compromises the reestablishment of the microtubule network after depolymerization and, thus, reduces primary cilium formation [29]. Also, USP14 controls ciliogenesis and cilia elongation through the downregulation of Hedgehog signal transduction, since USP14 inhibition positively affects the Hedgehog pathway [30]. On the other hand, *CYLD*, a tumor suppressor gene that encodes a deubiquitinating enzyme, causes cylindromatosis and is implicated in various signaling pathways. CYLD shows a specific localization at the centrosomes and the basal bodies, where it promotes ciliogenesis, and mutations in this gene cause cilia formation defects due to impaired basal body migration and docking [31]. Another DUB regulator is USP8, which deubiquitinates and stabilizes trichoplein, thus favoring ciliogenesis and counteracting the previously mentioned CUL3-KCTD17 ubiquitin ligase activity [32].

Not only the substrate/interacting partners of each protein but also the localization within the cilium organelle (whether at the basal body, the connecting cilium/transition zone, the axoneme, or the ciliary tip) (Fig. 13.1) and the precise cell cycle phase where the protein is being expressed are relevant for function [24]. The role of regulatory cilium proteins is different whether involved in anchoring the mother centriole to the membrane, microtubule organization and polymerization, ciliary trafficking, or cilium resorption.

13.5 UPS and Retinal Dystrophies

Several ubiquitin and SUMO pathway proteins participate in retinal development and photoreceptor differentiation, and mutations in the corresponding coding genes are causative of inherited retinal dystrophies (IRDs). A comprehensive analysis of the expression of DUB genes has been performed in the adult retina [19] and in fetal retinas of mouse and humans (Marfany et al., unpublished results). Moreover, and as already mentioned, many components of the ubiquitin-proteasome system are involved in the control of ciliogenesis and regulate cilium formation in photoreceptor cells, being potential candidates for causing a wide spectrum of ciliopathies as well as other disorders restricted to the retina.

Remarkably, the UPS genes involved in the regulation of ciliogenesis in cycling cells have been mostly associated with cancer but not to retinal disorders yet, most probably because mutations in these genes alter centrosome function and microtubule network organization, thus affecting multiple cell types and organs. Some clues on ubiquitin and SUMO pathway genes that particularly participate in retinal development and photoreceptor differentiation have been described in animal models. For instance, *fat facets* (FAF/USP9X) is a deubiquitinating enzyme that controls cell-to-cell communication and clathrin endocytosis in *Drosophila* photoreceptors. The FAF/USP9X mutant shows endocytosis dysregulation and ectopic photoreceptor determination and, thus, displays severe

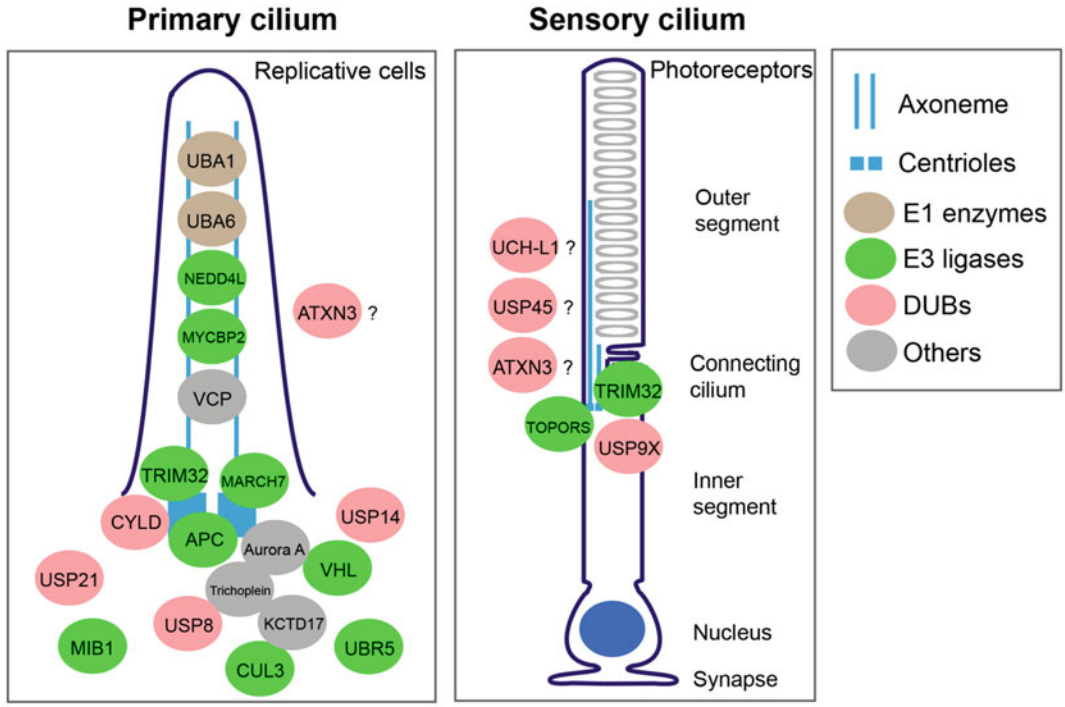

Fig. 13.1 Identified UPS proteins that regulate ciliogenesis in replicative and differentiated cells. Schematic representation of the localization of various UPS components into the cilium in the replicative versus differentiated cells (e.g., photoreceptors). Many of these proteins display different roles during ciliogenesis and, thus, show specific localization into the centrioles and/or axoneme of the cilium. In the sensory cilium, only E3 ligases and DUBs with a specific function in the retina are localized in the cilium, in contrast to the replicative cells, where proteins show cell cycle-dependent localization reflecting cilia formation/resorption

defects in photoreceptor differentiation [33]. *Usp5* deficiency in *Drosophila* eyes causes impairment in eye development. Loss of *Usp5* results in upregulation of Notch signaling and downregulation of RTK (receptor tyrosine kinase) signaling, leading to impaired photoreceptor development [34]. UCH-L1 is a DUB that participates in multiple pathways during eye development in *Drosophila*. Its overexpression in the eye imaginal disks induces a rough eye phenotype in the adult fly by downregulating the MAPK (mitogen-activated protein kinase) pathway [35]. On the other hand, the knockdown of DUB genes by morpholino injection in zebrafish embryos has identified *usp45* [36] and *atxn3* (Toulis et al. unpublished data) as causative of moderate to severe eye morphological defects, with defective formation of the retinal structures.

Apart from the role of UPS genes' role in retinal development and photoreceptor differentiation in animal models, mutations in several genes related to UPS in humans cause retinitis pigmentosa (RP), the most prevalent inherited retinal dystrophy (1:4000 people worldwide), and other inherited retinal disorders. Moreover, dysfunction of proteins of the UPS has been also associated with multifactorial retinal disorders, such as age-related macular degeneration, glaucoma, diabetic retinopathy, and retinal inflammation [37].

Among the UPS genes mutated in retinal disorders in humans, *KLHL7* encodes an E3 ubiquitin ligase of BTB-Kelch subfamily widely expressed in the retina, especially in rod photoreceptors. Different mutations in *KLHL7* have been associated with a late-onset form of autosomal dominant retinal degeneration that preferentially affects the rod photoreceptors, affecting both rod and cone electrophysiology [38–40]. On the other hand, biallelic mutations

in this gene cause a much severe recessive phenotype, the Crisponi syndrome (CS)/cold-induced sweating syndrome type 1 (CISS1)-like phenotype, with high neonatal lethality due to a developmental multi-organ disorder including early-onset retinal neurodegeneration [41]. The substrates of KLHL7 have not been determined, but its interaction with CUL3 suggests a direct or indirect proteostasis regulation of many CUL3 substrates related to ciliogenesis.

TOPORS stands out as one of the first genes related to UPS identified as a causative of inherited retinal dystrophies. TOPORS is a RING domain-containing E3 ubiquitin and SUMO dual ligase that localizes in the nucleus in speckled loci associated with promyelocytic leukemia bodies. Most notably, TOPORS localizes primarily to the basal bodies of photoreceptor sensory cilium connecting cilium and in the centrosomes and plays an important role in the regulation of primary cilia-dependent photoreceptor development and function, since its knockdown in zebrafish results in defective retinal development photoreceptor outer segment formation [42]. Point mutations, insertions, or deletions in TOPORS have been identified in different families explaining approximately 1% of autosomal dominant RP [43, 44], and it can be considered as a potential ciliopathy gene. However, no relevant function in non-retinal cilia has been reported yet for TOPORS.

Even though not directly related to ciliary function, mutations in the *PRPF8* gene in heterozygosis have been identified in Spanish families to cause adRP, most probably by haploinsufficiency [45]. PRPF8 is a pre-mRNA splicing factor participating in the dynamic assembly and dissociation of the spliceosome. PRPF8 displays the motifs of JAMM deubiquitinating zinc proteases and is usually grouped within this DUB group, but it is a catalytically inactive protein, since it lacks the residues that bind the metal ion required for activity.

In agreement with the retinal degeneration phenotype previously observed in the knockdown of *usp45* in zebrafish embryos [36], mutations in *USP45* have been also associated with retinal dystrophies in human patients. Whole-exome sequencing (WES) in Chinese families identified biallelic mutations within this gene implicated in the occurrence of Leber congenital amaurosis (LCA), an early and severe form of inherited retinal disorders, thus confirming the importance of USP45 in the maintenance of correct photoreceptor function [46]. The authors suggest a possible relation with ciliogenesis, even though no data are provided to support this hypothesis.

Again the localization of these proteins along the cilium organelle is extremely relevant for their function (Fig. 13.1). In the photoreceptor outer segment, which is a highly specialized cilium, intraciliary trafficking of the large amount of phototransduction and structural cargo proteins requires a highly regulated anterograde and retrograde transport. Therefore, a finely tuned control at the ciliary gate in the transition zone is paramount in photoreceptors; any transport disturbance may disrupt the cell homeostasis and, thus, trigger photoreceptor apoptosis. However, no UPS-related genes have been yet reported to regulate this key target for correct photoreceptor function.

It is remarkable that, although the retina is an extremely common organ affected in ciliopathies and many UPS proteins regulate ciliogenesis, in humans, mutations in only two UPS genes, which encode E3 ubiquitin ligases: *TOPORS,* for non-syndromic adRP, and *TRIM32 (BBS11),* for syndromic recessive BBS [47], have been undeniably associated with both ciliogenesis and retinal defects. Many research groups have addressed efforts in dissecting the relevance of proteins in controlling cell cycle and how their alteration results in cancer, whereas the identification of relevant proteins in neurosensory cilia mostly relies on mutations in human patients of rare diseases, which are clearly limited in number. Since proteomics and genetic analyses have identified more than 100 proteins involved in the formation of functional sensory cilia in the retina, and many of them are potentially controlled by ubiquitin and SUMO post-translational modifications, we hypothesize that the regulation landscape of photoreceptor ciliogenesis is still devoid of key UPS regulatory players (Fig. 13.1). Further work is required to unveil

novel E3 ligases and DUBs involved in cilia formation and ciliary trafficking and elucidate their precise function in photoreceptors, but with the application of highly precise gene editing techniques to generate specific mutants in cell and animal models, as well as in human organoids, we foresee a burgeoning field in the study of UPS in the regulation of ciliogenesis and its implications for elucidating the molecular basis of human disease.

13.6 Concluding Remarks

The retina is a complex neuronal tissue that requires a fine regulation at the transcriptional and protein levels. The ubiquitin-proteasome system participates in this regulation and we postulate that post-translational modifications, such as ubiquitination and SUMOylation, are implicated in the determination of photoreceptor cell fate, as well as retina development and ciliogenesis. Several reports have shown that some components of the UPS regulate the correct retinal function, especially in photoreceptors and sensory cilia. We postulate that further work will posit new E3 ligase and DUB genes as excellent candidates for either syndromic ciliopathies or non-syndromic retinal dystrophies.

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