The Roles of Phosducin-Like Protein 1 and Programmed Cell Death Protein 5 as Molecular Co-Chaperones of the Cytosolic Chaperonin Complex

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The Roles of Phosducin-Like Protein 1 and Programmed Cell Death Protein 5 as Molecular Co-Chaperones of the Cytosolic Chaperonin Complex

Christopher M. Tracy

A dissertation submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The Roles of Phosducin-Like Protein 1 and Programmed Cell Death Protein 5 as Molecular Co-Chaperones of the Cytosolic Chaperonin Complex

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Doctor of Philosophy

A fundamental question in biology is how proteins, which are synthesized by the ribosome as a linear sequence of amino acids, fold into their native functional state. Many proteins require the assistance of molecular chaperones to maneuver through the folding process to protect them from aggregation and to help them reach their native state in the very concentrated protein environment of the cell. This study focuses on the roles of Phosducin-like Protein 1 (PhLP1) and Programmed Cell Death Protein 5 (PDCD5) as molecular co-chaperones of the Cytosolic Chaperonin Complex (CCT).

Signaling in retinal photoreceptors is mediated by canonical G protein pathways. Previous in vitro studies have demonstrated that Gβ subunits rely on CCT and its co-chaperone PhLP1 to fold and assemble into Gβγ and RGS-Gβ5 heterodimers. The importance of PhLP1 in the assembly process was first demonstrated in vivo in a retinal rod photoreceptor-specific deletion of PhLP1. To test whether this mechanism applied to other cell types, we prepared a second mouse line that specifically disrupts the PhLP1 gene in cone photoreceptor cells and measured the effects on G-protein expression and cone visual signal transduction. In PhLP1 depleted cones, Gt2 and RGS9-Gβ5 levels were dramatically reduced, resulting a 60-fold decrease in cone sensitivity and a 50-fold increase in cone photoresponse recovery time. These results demonstrate a common mechanism of Gβγ and RGS9-Gβ5 assembly in rods and cones, underlining the significance of PhLP1/CCT-mediated folding in G protein signaling.

PDCD5 has been proposed to act as a pro-apoptotic factor and tumor suppressor. However, the mechanisms underlying its apoptotic function are largely unknown. A proteomics search for PhLP1 binding partners revealed a robust interaction between PDCD5 and CCT. PDCD5 formed a complex with CCT and β-tubulin, a key CCT folding substrate, and specifically inhibited β-tubulin folding. Cryo-electron microscopy studies of the PDCD5-CCT complex suggested a possible mechanism of inhibition of β-tubulin folding. PDCD5 binds the apical domain of the CCTβ subunit, projecting above the folding cavity without entering it. Like PDCD5, β-tubulin also interacts with the CCTβ apical domain, but a second site is found at the sensor loop deep within the folding cavity. These orientations of PDCD5 and β-tubulin suggest that PDCD5 sterically interferes with β-tubulin binding to the CCTβ apical domain and inhibits β-tubulin folding. Given the importance of tubulins in cell division and proliferation, PDCD5 might exert its apoptotic function at least in part through inhibition of β-tubulin folding.

Key words: Chaperonin, chaperone, G-protein signaling, phosducin-like protein, CCT, PhLP1, PDCD5, apoptosis
ACKNOWLEDGEMENTS

I wish to acknowledge all of the many people that contributed to this work. I especially want to thank Dr. Barry Willardson for the leadership and direction he provided me as I performed this research. I also thank my committee for their help and advice, as well as the Department of Chemistry and Biochemistry and the University for allowing me to pursue my doctorate here. I especially want to thank the wonderful people that I have worked with in the Willardson lab, particularly Amy Gray, Rebecca Plimpton, Jeffrey Lai, Tanner Shaw, and Devon Blake who directly contributed to this work. I also want to thank our collaborators Jorge Cuellar, Jose Valpuesta, Sasha Kolesnikov, and Vladimir Kefalov. Most importantly I want to thank my wife Miriam for her love, support, and dedication to our family and our three children Brigham, Andalynn, and Alora.
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ABBREVIATIONS

AF555  Alexa Fluor 555
BzF   p-benzoyl-L-phenylalanine
C-    Carboxyl terminus
CCT   Chaperonin containing tailless complex polypeptide 1
Cryo-EM  Cryo-electron microscopy
ERG   Electroretinogram
FLAG  FLAG octapeptide
G     Heterotrimeric GTP binding protein
GDP   Guanosine diphosphate
GFP   Green fluorescence protein
GGL   Gγ-like
GPCR  G-protein coupled receptor
GTP   Guanosine triphosphate
Gα    G-protein alpha subunit
Gβ    G-protein beta subunit
Gβγ   G-protein βγ subunit dimer
Gγ    G gamma subunit
HA    Hemagglutinin
HEK 293T  Human embryonic kidney 293 cells expressing T antigen
HSP   Heat-shock protein
IP     Immunoprecipitation
MS     Mass spectrometry
N-     Amino terminus
PBS   Phosphate-buffered saline
PCR   Polymerase chain reactions
Pdc   Phosducin
PDCD5 Programmed cell death protein 5
PhLP  Phosducin-like protein
PNA   Peanut agglutinin
RGS   Regulator of G-protein Signaling
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>SDS</td>
<td>Sodium dodecyl-sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TCP</td>
<td>Tailless complex polypeptide</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>U2OS</td>
<td>Homo sapiens bone osteosarcoma</td>
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CHAPTER 1:
CHAPERONE-MEDIATED ASSEMBLY OF G-PROTEIN COMPLEXES

Summary

G protein signaling depends on the ability of the individual subunits of the G protein heterotrimer to assemble into functional complexes. Formation of the G protein βγ (Gβγ) dimer is particularly challenging because it is an obligate dimer in which the individual subunits are unstable on their own. Recent studies have revealed an intricate chaperone system that brings the Gβ and Gγ subunits together. This system includes the cytosolic chaperonin containing TCP-1 (CCT) and its co-chaperone phosducin-like protein 1 (PhLP1). CCT assists Gβ in achieving its β-propeller structure, while PhLP1 releases Gβ from CCT and facilitates its interaction with Gγ. Once Gβγ is formed, PhLP1 remains bound until it is displaced by the Gα subunit and the G protein heterotrimer is brought together. Another obligate dimer is the complex between the G protein β5 subunit and a regulator of G protein signaling protein (RGS-Gβ5). RGS-Gβ5 also requires CCT for Gβ5 folding, but PhLP1 plays a different role. It stabilizes the interaction between Gβ5 and CCT, perhaps to increase folding efficiency. After Gβ5 folding PhLP1 must subsequently release, allowing the RGS protein to bind and form the RGS-Gβ5 dimer directly on CCT. RGS-Gβ5 is then freed from CCT to interact with its membrane anchoring protein and form a stable complex that turns off the G protein signal by catalyzing GTP hydrolysis on Gα.

Introduction

A fundamental question in biology is how proteins, which are synthesized by the ribosome as a linear sequence of amino acids, fold into their native functional state. It is now

* This chapter is a published review written by Dr. Willardson and me:
clear that many proteins require the assistance of molecular chaperones to maneuver through the folding process. Molecular chaperones are themselves proteins that protect newly synthesized or unfolded proteins from aggregation and help them reach their native state in the very concentrated protein environment of the cell (2).

**Chaperonins**

One important class of molecular chaperones is the chaperonins. Chaperonins are large, multi-subunit complexes that form stacked, double-ring structures with a central cavity in each ring. These cavities provide an isolated environment for client proteins to bind and fold (3,4). Each subunit consists of three domains: an equatorial domain that binds and hydrolyzes ATP, an apical domain that binds substrates, and an intermediate domain that connects the two other domains and facilitates inter-domain communication (4). There are two types of chaperonins. Group I chaperonins are found in bacteria (i.e. GroEL from *E. coli*), mitochondria and chloroplasts (Hsp60). Their ring structures are composed of seven identical subunits that bind and hydrolyze ATP. ATP binding is coordinated with encapsulation of substrates within the folding cavity by a small co-chaperone called GroES in *E. coli* and Hsp10 in eukaryotes (2,3).

The group II chaperonins are found in archeabacteria (named thermosomes) and in the eukaryotic cytosol (termed CCT, cytosolic chaperonin containing tailless complex polypeptide 1, also called TRiC). CCT is the most complex of all the chaperonins, with each of the two rings composed of eight paralogous subunits that orchestrate the folding of many proteins, with the most abundant substrates being actins and tubulins (4). In fact, CCT is required for folding 9-15% of all newly synthesized cytosolic proteins in the cell. CCT substrates tend to have complex domain topologies and range up to ~70 kDa in size (5,6). Nascent polypeptides or denatured proteins bind inside the folding cavity to regions of both the equatorial and apical domains of the
CCT subunits (7). The process of ATP binding and hydrolysis induces dramatic conformational changes in the apical domains that result in closure of the folding cavity by finger-like helical extensions found at the tip of the apical domains (8-13). The conformational change also exposes a more hydrophilic surface on the inside of the cavity (8,9). The substrate then folds in this sequestered environment. After phosphate release from the nucleotide-binding pocket, the apical domains reopen and the folded protein dissociates from CCT. If the protein has not yet reached its native structure, it can reassociate for another round of ATP binding and hydrolysis (8,9,11-13).

CCT substrates often require additional proteins called co-chaperones for efficient delivery or release from CCT. For example, the co-chaperone prefoldin is required for transfer of nascent actin or tubulin to CCT (14,15). The unfolded actin or tubulin binds to hydrophobic residues at the tips of the tentacle-like extensions of prefoldin (14). The complex then binds and transfers the nascent substrate to CCT to continue folding (14,16,17). Another CCT co-chaperone is Heat-shock Protein 70 (Hsp70), which besides acting as a chaperone on its own, can transfer different substrates to the chaperonin for more efficient folding (18). Co-chaperones can also mediate the release of proteins from CCT once they have completed folding. Phosducin-like Protein 1 (PhLP1) serves such a purpose in stably releasing the β subunit of the G-protein from CCT and thus plays a critical role in all of G protein signaling.

**G Protein Signaling**

Cells detect and respond to a myriad of extracellular signals via seven-transmembrane G protein-coupled receptors (GPCR) and their associated G protein signaling pathways (19). The list of molecules that transmit signals through GPCRs is impressive, including hormones, neurotransmitters, chemokines and sensory molecules such as odorants and tastants. Even
photons of light are detected by GPCRs and converted into a neural response by a G protein pathway in the photoreceptor cells of the retina (20). Such diverse signaling requires a large array of receptors, as seen by the nearly 900 genes encoding different GPCRs in humans (21). The importance of G protein signaling to human physiology is evidenced by the fact that GPCRs are the target of one third of currently marketed drugs (22).

The basic architecture of G protein signaling pathways is shown in Figure 1-1 (23-27). The pathway is initiated by the binding of the signaling molecule on the extracellular surface of the GPCR. This interaction causes a conformational change in the receptor which opens up its seven helical bundles on the intracellular surface, exposing the binding site for the G protein (28,29). In its inactive form, the G protein is a heterotrimer of α, β and γ subunits with GDP bound to the nucleotide binding site on the Ga subunit (25,30). Binding to the activated receptor releases GDP from Ga, allowing GTP to replace GDP in the nucleotide binding site (24). The extra phosphate of GTP induces a conformational change that disrupts the interaction of Ga with the Gβγ subunits, causing the dissociation of Ga-GTP from Gβγ (31,32). Ga-GTP is then free to interact with effector enzymes such as adenylyl cyclase, phospholipase Cβ or Rho guanine nucleotide exchange factors and regulate their activity (33). The Gβγ pair is an obligate dimer that remains together throughout its lifetime in the cell (34). Like Ga, Gβγ binds effectors enzymes (35-38) but can also interact with ion channels such as inwardly-rectifying K⁺ channels (39,40) and voltage-gated Ca²⁺ channels (41,42). The changes in activity of effector enzymes and ion channels lead to changes in the cellular concentration of second messengers such as cyclic nucleotides and Ca²⁺ as well as variations in the plasma membrane potential. These changes then drive the cellular response to the signal. The signal is turned off by the action of regulators of G
Figure 1-1. The G protein signaling cycle. The scheme depicts the G protein activation/inactivation cycle using the atomic structures of the individual components determined by X-ray crystallography. The entire cycle occurs on the plasma membrane with all components associated with the inner leaflet of the plasma membrane, or spanning the membrane in the case of the GPCR, and is only shown displaced from the membrane for clarity purposes. See text for a description of the cycle. Color code for the proteins is: GPCR – green, Gα – teal, Gβ1 – blue, Gγ – red, RGS DEP/DHEX domain – pink, Gβ3 – dark blue, RGS Gγ-like domain – dark red, RGS domain – orange. PDB numbers for the various structures are: GPCR-G protein complex (3SN6) (24), G protein heterotrimer (1GOT) (25), Gα-GTP (1TND) (26), Gβ5-RGS (1PB1) (27).

protein signaling (RGS) proteins that catalyze the hydrolysis of GTP to GDP on Gα, allowing the reassociation of Gα-GDP with Gβγ and returning the system to its inactive state (43).

In order for GPCRs, G proteins and effectors to perform these functions, they must first be synthesized, and their nascent chains must find their binding partners and move to the plasma membrane where the initial signaling events occur. Of the many diseases associated with malfunctions in G protein pathways, an important subset results from misfolding mutations in GPCRs or other pathway components that disrupt trafficking of the GPCR and assembly of the G protein and effector complexes (44-49). Thus, it has become increasingly important to
understand the folding pathways of the receptors and other components of the G protein pathway.

*Assembly of the Gβγ Dimer*

A particularly puzzling problem regarding the folding and assembly of G protein signaling components is how obligate oligomers such as the Gβγ dimer associate when the individual protomers of these protein complexes are not stable on their own. The protomers cannot simply wait after they have been synthesized by the ribosome for a random interaction with their binding partners to form a stable complex. This dilemma has been considered for some time for the Gβγ complex. Once formed, the dimer is very stable, but by itself the Gβ subunit is not (50). How then could the complex ever form if Gβ cannot fold into a stable structure on its own? Higgins and Casey in an early study of Gβγ assembly postulated that Gβ might require accessory proteins to associate with Gγ (50). Interestingly, Gβγ dimers could be formed in rabbit reticulocyte lysate (51) and in insect cells (50) where heterotrimeric G proteins are normally expressed, but not in bacteria where they are not (50), suggesting that any accessory proteins may have co-evolved with the G proteins to assemble the Gβγ dimer. It was some time later before additional clues began to surface and shed light on the mechanism of Gβγ assembly.

The first clues came from genetic studies of G protein signaling in the chestnut blight fungus *C. parasitica* in which disruption of a gene termed *bdm1* gave the exact same phenotype as disruption of the Gβ gene (52). This observation was interesting because the *bdm1* gene was homologous to phosducin, a known binding partner of Gβγ in mammalian photoreceptors. However, the absolute requirement for *bdm1* in G protein signaling in *C. parasitica* was inconsistent with the proposed role of phosducin in photoreceptors, which was to inhibit G protein signaling by binding Gβγ and blocking its interaction with Gα (53). Bdm1 is actually a
closer homolog of PhLP1, a member of the phosducin gene family with 65% homology to phosducin (54). PhLP1 also binds Gβγ like phosducin (55), but its expression pattern is very different, being found in most tissues and cell types, while phosducin is limited principally to photoreceptors (56). A breakthrough in this quandary came when our lab discovered that PhLP1 was a binding partner for the cytosolic chaperonin CCT (57). Our data suggested that PhLP1 was not a client protein of CCT, but a binding partner that bound above the folding cavity at the tips of the apical domains, similar to how GroES binds GroEL, leaving room within the protein cavity for a client protein (57,58). Although indirect, this finding was the first link between Gβγ and the protein chaperone system.

*Chaperone-mediated Assembly of G protein complexes*

While our work on the interaction between PhLP1 and CCT was on-going, several studies suggested that WD40 repeat proteins like Gβ were important CCT clients. A proteomic analysis of protein-protein interactions in yeast showed several interactions between yeast CCT and proteins containing WD40 repeats (59,60). WD40 repeats fold into β-propeller structures (30,61), a ring of β-sheets that often requires assistance from chaperones to correctly fold (6). Furthermore, several of these WD40 repeat proteins were found to require CCT for their folding (15,62). With the backdrop of these findings, a second genetic study, this time in *Dictyoselium*, shed further light on the issue. Like the previous genetic study, this work reported that genetic deletion of PhLP1 phenocopied a Gβ deletion (63). G protein signaling in these cells was lost in the absence of PhLP1 (63). Importantly, Gβ and Gγ could be expressed in the absence of PhLP1, but they did not associate with the plasma membrane as in the wild-type, suggesting that they were not forming Gβγ dimers (63). Putting all of this data together indicated to us that PhLP1 might be involved in Gβ folding and assembly with Gγ. We tested this hypothesis by measuring
the effects of siRNA-mediated depletion of PhLP1 in HEK-293 cells on the formation of nascent Gβγ dimers in a pulse-chase experimental format. A 70% knockdown of PhLP1 caused a 5-fold decrease in the rate of Gβγ assembly (64). Moreover, over-expression of an N-terminal truncation of PhLP1 completely blocked Gβγ assembly (64). This truncation was missing an important Gβ interaction site in helix 1 of PhLP1 (65), creating a variant that bound Gβγ poorly but still bound CCT well. As a result, the variant could displace endogenous PhLP1 from CCT, but without the ability to bind Gβ, it could not assist in Gβγ assembly. Thus, this PhLP1 variant was acting as a potent dominant negative inhibitor of Gβγ assembly. These experiments were consistent with our hypothesis that PhLP1 played an important role in Gβγ assembly and were supported by results from other labs using different experimental approaches (66,67).

Subsequent studies began to shed light on the mechanism by which PhLP1 assists in Gβγ assembly. Wells et al. showed that nascent Gβ bound CCT and was released in the presence of ATP and Gγ to form nascent Gβγ dimers, but no association of Gγ with CCT was observed, suggesting that Gβ was released from CCT upon association with Gγ (68). In a separate study, we found that PhLP1 and Gβ formed a ternary complex with CCT (69), and the stability of this complex was determined by protein kinase CK2 phosphorylation of PhLP1 at a unique triple serine sequence near the N-terminus (S18-20) (69). If S18-20 was phosphorylated, the ternary complex was short-lived, PhLP1 and Gβ were released from CCT and Gβγ dimers were formed (69). Maximal Gβγ assembly activity required two or three phosphorylation events within the S18-20 sequence (69). If S18-20 was not phosphorylated the complex was stable, PhLP1 and Gβ were not released from CCT and Gβγ dimer formation was severely impaired (69). Thus, CK2 phosphorylation of PhLP1 triggers the release of Gβ from CCT, allowing its association with Gγ (69).
From these results, a model for PhLP1-mediated Gβγ assembly can be put forward (Figure 1-2A) (70). In this model, nascent Gβ associates with CCT shortly after its synthesis on the ribosome. The process by which nascent Gβ is delivered to CCT has not been studied. One possibility is that co-translational chaperones of the Hsp70 and Hsp40 families bind the nascent Gβ during its synthesis and then transfer the nascent chain to CCT. Such a relay system of chaperones has been described for other proteins (71). Another possibility is that CCT accepts the nascent Gβ chain directly from the ribosome during synthesis. Direct binding of nascent chains to CCT has been observed previously for actin (72). Whatever the mechanism of delivery to CCT, once Gβ binds it is then folded by CCT. Recent cryo-EM studies of the Gβ-CCT intermediate from Jose Valpuesta’s lab indicate that Gβ achieves a near native conformation within the folding cavity (73). However, unlike other CCT substrates that are released once they have achieved a stable fold, Gβ does not release from CCT in the absence of PhLP1 (69).

![Figure 1-2. Effects of PhLP1 deletion on Gβγ and RGS9-Gβ5 assembly.](image)

A, B, The scheme summarizes the proposed mechanisms for G-protein heterotrimer (A) and R9AP/RGS9-Gβ5 assembly (B) and the effects of PhLP1 deletion on the assembly process (shown in red). Reprinted from (70)
inability to release most likely stems from the fact that the Gβ β-propeller is unstable on its own. The role of PhLP1 may be to bind and stabilize the β-propeller, thereby reducing the contacts between Gβ and CCT and facilitating release.

**Role of PhLP1 phosphorylation in Gβγ assembly**

Another unresolved question in this model is why phosphorylation of PhLP1 is required for Gβ release from CCT. CK2 phosphorylation does not affect PhLP1 binding to Gβγ, however it does increase the binding of PhLP1 to CCT significantly in the absence of Gβ (69). This increase in binding upon PhLP1 phosphorylation seems contrary to the observed increase in the rate of release of PhLP1-Gβ from CCT upon PhLP1 phosphorylation (69). A possible explanation could be that phosphorylation changes the orientation of PhLP1 on CCT. The basis of this notion comes from cryo-EM studies of the PhLP1-CCT complex. Unphosphorylated PhLP1 bound CCT in two different orientations, one in which the N-terminal domain of PhLP1 was oriented toward the CCTα subunit and a second in which it was oriented toward the CCTβ subunit (58). The first orientation was favored, being populated by two-thirds of the complexes. However, the cryo-EM structure of PhLP1-Gβ-CCT shows Gβ bound to CCTβ within the folding cavity and PhLP1 sitting above Gβ, spanning the cavity (73). Thus, to effectively interact with Gβ, PhLP1 must be oriented toward CCTβ in the second conformation. Perhaps phosphorylation favors the second orientation and puts PhLP1 in position to bind Gβ. Once bound, both PhLP1 and Gβ could lose contacts with CCT and subsequently release from the chaperonin. Additional structural studies of both phosphorylated and unphosphorylated PhLP1 bound to CCT will be needed to test this possibility.

It is currently unclear whether CK2 phosphorylation of PhLP1 is regulated. CK2 is a constitutively active kinase which is controlled only by its expression levels and the proximity of
its many substrates, which in turn is determined by its subcellular localization and the complexes in which it participates (74). Thus, it is possible that PhLP1 is constitutively phosphorylated and the unphosphorylated form is rare. Fortunately, this possibility can be readily tested because the phosphorylation status of PhLP1 is easily measured by following the decrease in the mobility of PhLP1 in SDS-PAGE that occurs upon CK2 phosphorylation at S18-20 (69,75). In HEK-293 cells, mouse embryonic tissue and mouse heart, PhLP1 was 100% phosphorylated at S18-20, while it was 70-80% phosphorylated in mouse brain and 10-20% phosphorylated in mouse adrenal gland (75). These results suggest that CK2 phosphorylation of PhLP1 S18-20 is regulated differently in different tissues and might be a means of controlling the rate of Gβγ assembly.

**Mechanism of Gβ association with Gγ**

The model of Figure 1-2 predicts that Gβ is released from CCT in a complex with PhLP1 prior to its interaction with Gγ. There are several observations that support this idea. First, nascent Gβ was shown by co-immunoprecipitation to form a complex with PhLP1 that did not contain Gγ (64). Thus, nascent Gβ must be binding PhLP1 prior to its association with Gγ. This co-immunoprecipitated complex could have been either a PhLP1-Gβ dimer or a PhLP1-Gβ-CCT ternary complex. Second, Gγ does not associate with CCT (68,69), indicating that Gβ must be released from CCT prior to its association with Gγ. Third, the rate of Gβ release from CCT was increased more than 3-fold in the presence of PhLP1 and that rate was not increased further by addition of Gγ (69). These findings are all consistent with a release of PhLP1-Gβ from CCT prior to its association with Gγ. This said, we have not been able to purify PhLP1-Gβ complexes, suggesting that the complex is short-lived. It seems likely that Gγ binding occurs very quickly after PhLP1-Gβ is released from CCT.
Thus far, we have not addressed the fate of G\gamma after its synthesis and prior to its association with G\beta. G\gamma can be expressed in cells in the absence of G\beta, although the expression level is much less than in the presence of G\beta (69). Little is known about the fate of G\gamma after its synthesis by the ribosome. It is unlikely that G\gamma folds into a stable structure in the absence of G\beta (50), thus G\gamma is a good candidate for chaperone assistance. In fact, Dupre et al. have reported an interaction between the J-domain containing chaperone, DRiP78 and G\gamma. DRiP78 bound G\gamma and protected it from degradation (76). They also saw an interaction between PhLP1 and DRiP78 and suggested that this interaction facilitates the transfer of G\gamma to G\beta bound to PhLP1. It is important to note that G\gamma binds the opposite face of G\beta as PhLP1, so their binding sites do not overlap (65). As a result, G\gamma could readily associate with G\beta while PhLP1 is still bound. Once formed, G\beta\gamma is a very stable dimer that can only be dissociated by denaturation (34). In the dimer, the C-terminal CaaX motif of the G\gamma subunit is prenylated with either a farnesyl or a geranylyl isoprenoid, greatly increasing the affinity of G\beta\gamma for lipid bilayers (77).

**Association of G\beta\gamma with Ga**

Association of G\beta\gamma with Ga is believed to occur on the cytoplasmic face of the endoplasmic reticulum membrane (77). In the process, PhLP1 must be released from G\beta\gamma because the PhLP1 binding site on G\beta\gamma overlaps with both the Ga binding face and the membrane binding surface of G\beta\gamma (65). Furthermore, Ga has a much stronger affinity than PhLP1 for G\beta\gamma (78,79). This prediction was confirmed experimentally as Ga\alpha_{i3} was found to displace PhLP1 from nascent G\beta\gamma in co-expression experiments in HEK-293 cells (64).

Substantially less is known about the course of Ga prior to its association with G\beta\gamma, though studies suggest it may also require chaperones to facilitate its folding and/or assembly with G\beta\gamma. One study showed that transducin α (Ga\alpha) co-immunoprecipitates with CCT when
expressed in reticulocyte lysates (80), suggesting that Gα subunits may also be a CCT substrate. Additionally, CCT-bound Gαt was more susceptible to protein degradation and was released from CCT with the addition of ATP, both characteristics of other CCT substrates. Moreover, reticulocyte translated Gαt could be activated by light-activated rhodopsin, indicating that the Gαt had reached its native conformation. Comparable assays in rat retinal rod cells showed a similar association of Gαt with CCT (80). Currently, no other evidence links CCT to the folding or assembly of other Gα subunits, though CCT’s role in Gα folding certainly warrants further investigation.

Another potential chaperone of Gα subunits is the non-GPCR guanine-nucleotide exchange factor (GEF) resistance to inhibitors of cholinesterase 8 (Ric-8). Ric-8A stimulates the exchange of GTP for GDP for Gαi, Gαq and Gα12/13, while Ric-8B acts as a GEF for Gαs (81,82). Genetic studies in C. elegans (83) and D. melanogaster have implicated Ric-8 in G protein regulation of centrosome movements during cell division (84-86). In C. elegans, Ric-8 is required for cortical localization of GPA-16, a Gα homolog (87), and loss-of-function Ric-8 mutants in Drosophila resulted in defects in gastrulation, neuroblast differentiation, spindle orientation, and asymmetric division (84-86). Upon further investigation, these Drosophila studies showed that Gαi, Gαo, and Gβ did not localize to the plasma membrane in Ric-8 mutants, but rather to the cytosol. Moreover, the steady state levels of Gαi and Gβ were also reduced in Ric-8 mutants compared to wild type, and Gαi did not associate with Gβ in Ric-8 mutants (84-86). Likewise, Ric-8B promotes expression of Gαolf and Gαs in mammalian cultured cells (88,89), and Ric-8A or Ric-8B co-expression with Gα subunits in insect cells greatly increases Gα levels (90). Recent gene-deletion studies of Ric-8A and Ric-8B show dramatic effects on the expression of Gαi, Gαq and Gα13 or Gαs, respectively, which resulted in pleiotropic G protein
signaling defects (91). Ric-8 gene deletion also resulted in less efficient localization of G proteins to the plasma membrane and increased degradation (91). Studies using in vitro translation systems strengthen the chaperone hypothesis. Gαs translated in Ric-8 deplete RRL were more susceptible to limited trypsinization compared to mocked deplete RRL or recombinant Ric-8-supplemented RRL, suggesting that Gα is not properly folded in the absence of Ric-8 (92). Similar experiments from wheat germ extract, which does not contain any Ric-8 homolog, showed similar results (92). Together, all these studies suggest that Ric-8 binds to Gα as a chaperone to stabilize the subunit and facilitates its association with βγ subunits. Once the Gαβγ heterotrimer is formed, Gα is palmitoylated and the heterotrimer is trafficked to the plasma membrane, most likely in a complex with a GPCR (77).

Specificity of PhLP1-mediated Gβγ assembly

Most of the work on Gβγ assembly has focused on the Gβ1γ2 dimer, a common Gβγ pair. However, there are five Gβ isoforms and twelve Gγ isoforms. Gβ1 and Gβ4 can form dimers with all Gγs, while Gβ2 and Gβ3 are more selective (93). In contrast, Gβ5 does not interact with Gγs in vivo but instead forms dimers with the regulator of G protein signaling (RGS) subfamily 7, which consists of RGS6, 7, 9 and 11 (94). These RGS proteins have a Gγ-like (Ggl) domain that binds Gβ5 very similarly to the binding of Gγ to the other Gβs (27), and they play an important role in turning-off G protein signals in neuronal cells (94). An important question that arose from the Gβγ assembly work was whether PhLP1 and CCT were involved in the formation of all Gβγ and Gβ5-RGS combinations or just a subset. A related question was whether PhLP1 and CCT contributed to the specificity of Gβγ interactions by determining which Gβ subunits interacted with which Gγ subunits. To address these questions, we performed a comprehensive study of the role of PhLP1 in the assembly of the various Gβγ and Gβ5-RGS combinations. In co-
immunoprecipitation experiments, we found that PhLP1 bound all five Gβ subunits. Binding of Gβ1-4 to PhLP1 was similar, while binding of Gβ5 to PhLP1 was ~4-fold weaker than the other Gβs. Furthermore, the assembly of Gβ1-4 with Gγ2 was inhibited by 70-80% by a 70% siRNA-mediated PhLP1 knockdown (95). Similarly, over-expression of the dominant negative PhLP1 Δ1-75 variant reduced assembly of Gβ1-4 with Gγ2 by 80-90%. These results show that PhLP1 assisted in the assembly of all four Gβs that form dimers with Gγs. In the reciprocal experiment, dimer formation between Gβ2 and all twelve Gγ subunits was inhibited by ~80% upon PhLP1 knockdown or PhLP1 Δ1-75 over-expression (95). Another study showed that Gβ1-4 all bound CCT and that CCT was required for Gβ1γ2, Gβ1γ3, and Gβ2γ3 assembly (68). Together, these results indicate that PhLP1 and CCT are required for assembly of all Gβγ combinations.

Our lab also studied the effect of PhLP1 on the specificity of Gβγ dimer formation (95). Gβ2 was used in this study because it forms dimers with some Gγs but not others. The data reflect this observation. Gβ2 dimer formation was strong with Gγ subfamily II (Gγ2,3,4 and 8), moderate with Gγ subfamily III (Gγ7, 12), and generally weak with subfamilies I (Gγ1, 9 and 10) and IV (Gγ5, 10) and not observed with subfamily V (Gγ13) (95). Inhibition of PhLP1 activity by siRNA knockdown or PhLP1 Δ1-75 over-expression did not change this order of Gβ2Gγx specificity at all, but consistently inhibited dimer formation by ~80% no matter the original extent of Gβγ dimer formation (95). This result shows that PhLP1 does not influence the specificity of Gβγ interactions, but simply facilitates association of Gβγ dimers that are intrinsically stable. Furthermore, the fact that the stability of Gβ2γx dimers segregates so nicely into Gγ subfamilies shows that the specificity is inherent in the complementarity of the binding surfaces between Gβ2 and the Gγ subunits as determined by the amino acid sequences of the Gγ subunits themselves (95).
Assembly of the RGS-Gβ3 dimer

The structure of the RGS9-Gβ5 dimer shows that the Ggl domain of RGS9 occupies the same position on the Gβ3 β-propeller as does Gγ1 on the Gβ1 β-propeller (Figure 1-3) (27,65). This structural similarity suggests that Gβ5-RGS dimers may be assembled by a similar mechanism as Gβγ dimers. However, Gβ5 binds both CCT and PhLP1 weaker than Gβ1 (68,69), suggesting that there are significant differences between Gβ5-RGS and Gβγ assembly. To address this issue, we assessed the contribution of PhLP1 and CCT to the formation of the Gβ5-RGS7 dimer in HEK-293 cells (95). An 80% PhLP1 siRNA knockdown caused a 2-fold reduction in the rate of Gβ5-RGS7 assembly. This decrease was less than the 5-fold decrease seen with Gβ1γ2 assembly (64,69). In the case of CCT, a 50% siRNA knockdown caused a 2-fold reduction in the rate of Gβ5-RGS7 assembly as well as a 2-fold decrease in Gβ1γ2 assembly (95). From these results, it appears that Gβ5-RGS7 assembly is as dependent on CCT as is Gβγ assembly, but it may be less dependent on PhLP1. We went on to carry out a series of additional experiments to try to determine what might be different about the role of PhLP1 in Gβ5-RGS7 assembly. First, we looked at the effects of over-expression of PhLP1 on the rate of Gβ5-RGS7 assembly. Surprisingly, PhLP1 over-expression caused a small ~25% decrease in the rate of Gβ5-RGS7 assembly. This decrease is compared to a 4-fold increase in Gβ1γ2 assembly upon PhLP1 over-expression (64,69). Second, we measured the effects of PhLP1 over-expression on the binding of Gβ5 to CCT. There was a striking 10-fold increase in the amount of Gβ5 associated with CCT upon PhLP1 over-expression whether RGS7 was co-expressed or not (95). This effect is in stark contrast to the observed release of Gβ1 from CCT upon PhLP1 over-expression (69).
These differences in the contribution of PhLP1 to Gβ5-RGS and Gβγ assembly may be explained by differences in the binding of PhLP1 to the two dimers. PhLP1 binds Gβ1γ2 with a 100 nM binding affinity and Gβ5γ2 with a 440 nM binding affinity, while it shows no measurable binding to RGS9-Gβ5 (95). The structural basis for these binding differences can be seen by examining the crystal structures of phosducin-Gβγ and RGS9-Gβ5 complexes (Figure 1-3). As mentioned above, phosducin binds Gβγ on the same face as Ga, which is on the opposite side of the β-propeller as the Gγ binding face (65). With its homology to phosducin and similar binding contacts (54,64,66), PhLP1 would be expected to bind Gβγ like phosducin. Thus, Gγ can associate with Gβ while PhLP1 is bound. This is not the case with RGS9-Gβ5. Its structure shows that the N-terminal lobe (the DEP/DHEX domain) of RGS9 interacts with the same face of Gβ as PhLP1(27). This overlap in the binding sites must preclude the formation of a PhLP1-Gβ5-RGS9 complex. As a result, PhLP1 must be released from Gβ5 before an RGS protein can bind.

A model for Gβ5-RGS assembly which is consistent with current data is presented in Figure 1-2B. This model has important similarities and differences to the Gβγ assembly model. In both models, Gβ1 and Gβ5 require CCT for their folding. However, PhLP1 does not promote the release of Gβ5 from CCT as it does for Gβ1; rather it stabilizes the interaction, presumably to
enhance folding of Gβ5. Moreover, PhLP1 must be released from Gβ5 prior to its association with the RGS protein, whereas it may remain associated with Gβ while Gβ binds Gγ. This need for PhLP1 in Gβ5 folding coupled with the requirement for release of PhLP1 prior to Gβ5-RGS assembly allows for both the observed decrease in Gβ5-RGS7 assembly when the cellular level of PhLP1 is decreased in the siRNA experiments and also the observed decrease in assembly when PhLP1 is increased in the over-expression experiments. The model goes on to predict that the RGS protein associates with Gβ5 while it is still bound to CCT, again in contrast to the Gβγ assembly model in which Gβ must be released from CCT prior to association of Gγ. This prediction is based on the observation that Gβ5 recruits RGS7 to CCT, creating an RGS7-Gβ5-CCT ternary intermediate (95). The nascent RGS protein may be delivered to Gβ5 on CCT by the Hsc70 chaperone given that Hsc70 is known to deliver folding clients to CCT (18) and an interaction between Hsc70 and RGS7 has been reported previously (96). Once the Gβ5-RGS7 dimer is formed on CCT, it can then be released to interact with its membrane anchoring protein. Only then is the complex fully stabilized and able to carry out its function in accelerating GTP hydrolysis on Ga subunits (97).

**Conclusion**

Research over the past decade has yielded considerable insight into the mechanism of assembly of the obligate dimers of Gβ subunits with Gγ subunits and RGS proteins. The data point to PhLP1 as an important co-chaperone with CCT in the folding and assembly of all complexes containing Gβ subunits. Yet there are many questions that remain. For example, all of the work on the mechanism of dimer assembly has been done in cell culture and thus needs to be examined in vivo. Toward this end, we have prepared conditional knockout mice of Phlp1 in retinal rod and cone cells which are described in Chapter 2. Another question is whether PhLP1
serves as a co-chaperone for other proteins or performs any other functions. In seeking to answer this question, we serendipitously discovered a novel regulator of β-tubulin folding by CCT, the programmed cell death protein 5 (PDCD5), which is discussed further in Chapter 3.

Finally and perhaps most importantly, can this information be exploited to create new drug targets to modulate G protein signaling at the level of G protein complex assembly? Could small molecule inhibitors or enhancers of the PhLP1-Gβ interaction be used to control the amount of Gβγ and Gβ5-RGS dimers made by the cell? The RGS proteins themselves have been forwarded as a good therapeutic target (98). It seems that targeting the assembly of Gβγ and Gβ5-RGS dimers is also a promising avenue of translational research to explore. Such targeting molecules could be useful pharmacological tools in the treatment of some of the many diseases associated with malfunctions in G protein signaling.
CHAPTER 2:
CHARACTERIZATION OF PHOSDUCIN-LIKE PROTEIN 1 GENE DELETION IN CONE CELLS OF THE MOUSE RETINA

Summary

*Signaling in retinal photoreceptors is mediated by canonical G protein pathways. Previous in vitro studies have demonstrated that Gβ subunits rely on CCT and its co-chaperone PhLP1 to fold and assemble into Gβγ and RGS-Gβ5 heterodimers. The importance of PhLP1 in the assembly process was demonstrated in vivo in a retinal rod photoreceptor-specific deletion of PhLP1. To test whether this mechanism applied to other cells, we prepared a second mouse line that specifically disrupts the PhLP1 gene in cone photoreceptor cells and measured the effects on G-protein expression and cone visual signal transduction. In PhLP1 depleted cones, Gβ3 levels were dramatically reduced, resulting in ~70% decrease in the levels of its Ga2 and Gγ8 binding partners. This decreases caused an ~10-fold decrease in cone photoreceptor sensitivity in full-field electroretinograms (ERGs) and a 60-fold decrease in transretinal ERGs. RGS9-Gβ5 complexes were also substantially reduced as shown by immunofluorescent staining and resulted in a 50-fold increase in cone photoresponse recovery time. These results demonstrate a common mechanism of Gβγ and RGS9-Gβ5 assembly in rods and cones and suggest that PhLP1 and CCT-mediated assembly of these complexes is shared in other cells, underlining the significance of these chaperones in G protein signaling.

Introduction

Phototransduction is the process by which light activates an electrical response in photoreceptor cells. Vertebrates rely on two types of photoreceptors: rods and cones. Rods

*I performed the work represented in Figures 3-3–3-6 with the help of Devon Blake, a spectacular undergraduate in our lab. The work in Figure 7 was performed by Alexander Dr. Kolesnikov of the Kefalov lab at the Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO.
function in low light conditions, whereas cones are specialized for brighter light conditions. Signaling in both photoreceptors is mediated by canonical G protein signaling. The GPCR in rods is rhodopsin. There are multiple cone opsins and the number varies for each species. Humans have three which correspond to long (red ~557 nm), middle (green 530 nm), and short (blue 426 nm) wavelengths. Mice only have two: middle with maximum sensitivity at 508 nm and short with maximum sensitivity at 360 nm (99).

Both rods and cones have four primary structural regions: the outer segment, inner segment, cell body and synaptic terminus (see Figure 2-1). The outer segment is composed of densely packed membrane discs that house all the molecular components for phototransduction, including the GPCRs, which serve as a major structural component of the discs. The inner segments contain the ER and mitochondria and provide the high amount of energy needed for phototransduction. The cell body contains the nucleus, and the synaptic terminus transmits the signal from the photoreceptor to the bipolar cells.

In the absence of light, there is a constant current of inward flowing sodium and calcium cations that maintain the photoreceptor in a depolarized state, constantly releasing the inhibitory neurotransmitter glutamate onto the downstream bipolar cells. This steady cation conductance is referred to as the “dark current.” Upon light stimulation, the ion channels are closed, leading to hyperpolarization of the photoreceptor and termination of glutamate release. The decrease in
glutamate release is detected by the downstream bipolar cells. Then the signal is amplified and transmitted to the brain.

Most vertebrate species use the chromophore 11-cis-retinal as a ligand covalently bound to visual GPCRs (99,100). 11-cis-retinal acts as an antagonist to the GPCR until it absorbs a photon of light upon which it isomerizes to all-trans retinal, a powerful agonist to the receptor. This isomerization causes a dramatic conformational change in the receptor, opening its intracellular domain and allowing binding of the α subunit of transducin, the G protein ($G\alpha_t$) responsible for signaling in photoreceptors. The bound $G\alpha_t$ then exchanges its GDP for GTP and dissociates from the receptor whereupon it binds and activates a phosphodiesterase (PDE). PDE activation causes the intracellular cGMP concentration to decrease, which closes cGMP-gated ion channels and results in membrane hyperpolarization and termination of glutamate release. The mechanism of rod photoreceptor G-protein signaling is shown in Figure 2-2 (101).

Rod physiology and phototransduction is better understood because rods are more easily isolated than cones. Additionally, cone studies in mouse models are complicated by the relatively low cone abundance in the retina, with cones representing only 3% of the photoreceptors (99). Despite their low abundance, advancements in gene targeting technology have allowed researchers to more effectively study cone signaling in the mouse (99). One study showed that knockout of a neural leucine zipper transcription factor (Nrl-
/-) alters cell fate so that rods develop into cone-like receptors (102). A second study showed that deletion of the Ga\textsubscript{i} gene (GNAT\textsuperscript{−/−}) blocks all phototransduction through the rods without causing photoreceptor degeneration, unlike many other knockout mice targeting rod phototransduction components (103). Lastly, a line expressing EGFP in cones facilitates their easy identification (104).

The photoreceptor serves as a great model for studying G-protein signaling \textit{in vivo}. Consequently, our lab sought to develop a conditional knockout mouse of PhLP1 to confirm the proposed mechanisms of PhLP1-mediated G\textbeta\gamma assembly based on our previous work \textit{in vitro} and in cell models (58,64,69,95). We engineered a conditional knock-out mouse in rod photoreceptors using the Cre-lox selection method, driven by the rhodopsin promoter (70). Specific deletion of PhLP1 caused a dramatic decrease in the levels of rod G\textsubscript{i} subunits as compared to WT animals, despite equal levels of the corresponding mRNA. These dramatic decreases are directly attributed to the inability of G\textbeta and G\textgamma to form heterodimers, as show by G\textbeta\gamma isolation from rod outer segment purifications and whole retina extracts. The loss of G\textbeta\gamma dimers resulted in a four-fold decrease of G\textalpha\textsubscript{1}. Interestingly, G\textbeta was decreased by only 68%, even though no G\textbeta\gamma dimers were detected. Co-immunoprecipitations showed an increase of G\textbeta bound to CCT in PhLP-deleted animals compared to controls. This increase suggests that, in the absence of PhLP1, G\textbeta is stabilized through accumulation on CCT. This accumulation likely caused the progressive retinal degeneration seen in the PhLP1 knockout mice. Additionally, PhLP1 deletion severely impaired visual behavior and electrophysiological responses. Perhaps the most novel finding was how crucial PhLP1 was in the assembly of RGS9-G\textbeta\textsubscript{5} complexes. Our previous studies with RGS7 in cultured cells suggested only partial dependence on PhLP1 for complex assembly (95), whereas in PhLP1 knockout rods both G\textbeta\textsubscript{5} and RGS9 expression
was decreased by 95%. This depletion completely matched the slow rate of photoresponse recovery measured in both dim and saturating flashes (70).

With the findings of the PhLP1 rod knockout, the natural progression would be to investigate the same effects in cones. PhLP1 deletion in cones would test whether the mechanism of Gβγ and RGS9-Gβ5 assembly was shared between rods and cones. Cone transducin consists of Gαt2 and Gβ3γ8 while rod transducin is made up of Gαt1 and Gβ1γ1. Thus, a PhLP1 deletion in cones would show whether Gβ3γ8 requires PhLP1 for its assembly and would provide an in vivo test of the hypothesis, formulated from studies of Gβγ assembly in cell culture (95), that PhLP1 is required for assembly of all Gβγ combinations. In addition, deletion of PhLP1 in the cones could provide new understanding of the differences between rod and cone signaling mechanisms. Despite their similar functions, there are two fundamental differences between rods and cones. Cones are less sensitive to light than rods (99, 105), and cones have faster photoresponse recovery rates than rods (99, 105). The rate-limiting step in rod photoresponse recovery has been shown to be the RGS9-Gβ5-catalyzed hydrolysis of GTP by Gαt1 (106). Cones are believed to express more RGS9-Gβ5 than rods, which could explain the faster recovery rates in cones (107). However, this notion has not been tested experimentally. If PhLP1 deletion in cones were to result in loss of RGS9-Gβ5 as seen in rods, then the importance of RGS9-Gβ5 in cone photoresponse recovery could be directly assessed. For these reasons, we prepared and characterized a mouse line with a cone-specific PhLP1-deletion.

**Experimental Procedures**

*Development of cone phlp1 gene deletion*

All experiments with mice were performed in accordance with National Institutes of Health policy on animal use and were approved by the Brigham Young University and
Washington University Institutional Animal Care and Use Committees. Generation of the Phlp1-loxP mouse was described previously (70). Mice containing the Phlp1-loxP were bred with the human red/green pigment (HRGP) gene promoter (108) to achieve conditional knockout of the Phlp1 gene in cone photoreceptors. PCR detection of the cre and Phlp-loxP genes were carried out using the appropriate primers (HRGP: 5'-AGG TGT AGA GAA GGC ACT TAG C-3' and 5'-CTA ATC GCC ATC TTC CAG CAG G-3'; PhLP-loxp 5' GAT CAC TTT GAC TGG GGA ATG ATT TTA GGT 3' and 5' GAG GTG GTA AGC AGG TGT ACT GGC TGG TTT 3').

Antibodies

The following antibodies used in this study. Primary antibodies: PhLP1 (109), Gαt1 and Gγ1 (Santa Cruz), Gα2 and Gγ8 (110), Gβ1 (111), Gβ3 (Sigma), Gβ5 (Proteintech), cone opsin (Millipore), cone arrestin (112), RGS9-1 (113). Secondary antibodies: FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories), TRITC-conjugated peanut agglutinin (Vector Laboratories), AF555-conjugate goat anti-rabbit (Life Technologies).

Immunohistochemistry and assessment of photoreceptor degeneration

The expression levels of PhLP1 and other visual signaling proteins in cone photoreceptors were tested by immunocytochemistry as described previously (70). Briefly, superior hemisphere of eyes from 30-40 day-old PhLP1F/FCre+ and control mice were cautery-marked for orientation. These eyes were enucleated under ambient illumination without adaptation and were immersion-fixed for 1-2 hr. using freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and cryo-protected overnight in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). The cornea and lens were then removed, and the eyecups were embedded in optimal cutting temperature (OCT) compound and cryo-sectioning was performed. Cryo-sections of 12 μm were cut through the optic nerve head along the vertical meridian and
were placed on superfrost microscope slides. For immunohistochemistry, sections were rinsed in 0.1 M phosphate buffer, and blocked for 1 h using either 10% donkey serum or 10% normal goat serum, 0.1% Triton X-100 in 0.1 M phosphate buffer (pH 7.4). Primary antibodies to PhLP1 (1:100 dilution), Gaα2, Gβ3 (1:200), Gγ8 (1:50), cone opsins (1:50), RGS9-1 (1:100), or Gβ5 (1:50), were applied to each group of four sections in a humidified chamber overnight at 4°C. After rinsing in three 10-min phosphate buffer washes, fluorescein isothiocyanate-conjugated secondary antibodies at a 1:200 dilution, AF555-conjugated secondary antibodies at a 1:1000 dilution or TRITC-conjugated peanut agglutinin at a 1:200 dilution were applied for 1-2 h at room temperature in a light protected, humidified chamber. The sections were viewed using an Olympus FluoView FV1000 confocal laser scanning microscope with a 60x, 1.4 numerical aperture oil objective lens and an optical slit setting of < 0.9 μm. Images were taken consistently inferior to the optic nerve of each section.

To aid in proper immunolocalization of signaling proteins that are expressed in both rods and cones (PhLP1, RGS9-1, Gβ5) immunohistochemistry experiments were performed on HRGP mice bred with enhanced green fluorescent protein (EGFP) cone-expressing mice (104). The Phlp-loxP allele was then bred in to create a knockout that expressed EGFP in the cones.

Cryo-sections with intact morphology were used for further analysis to determine photoreceptor degeneration by staining with TRITC-conjugated PNA to determine relative cone size and number at mice of 1 to 9 months.

**Determination of retinal protein expression**

Whole retina extracts were prepared from eyes of 30–40 day-old PhLP1
+ mice and controls under ambient illumination. These retinas were harvested and placed in ice-cold RIPA buffer (phosphate buffered saline with 1% NP-40 and 6 μl/mL Sigma Protease inhibitor
cocktail). The retinas were then passed through an 18G needle 20 times and a 25G needle 10 times to release the proteins. Extracts were centrifuged at 13,800 rpm for 10 min at 4°C to remove cellular debris. Protein concentrations were determined by BCA protein assay, and extracts with equal amounts of protein were loaded and resolved on 10% or 14% Tris-glycine-SDS gels or 16.5% Tricine-SDS gels and transferred onto nitrocellulose membranes using an iBlot transfer apparatus (Invitrogen). After blocking with LICOR Blocking buffer for 1 h, membranes were immunoblotted for each of visual proteins as indicated. The amounts of each protein in the immunoblots were quantified using a LICOR Odyssey near-infrared imaging system and compared to controls.

Quantitative RT-PCR

Total RNA from whole retinas was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Approximately 2 μg of the isolated RNA was then treated with DNase (Promega) and reverse transcribed using dT oligo primers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions to synthesize cDNA. The RT-PCR was performed using the Taq-Man gene expression assay primers and probe mix (Applied Biosystems). Mouse Actb (Mm00607939_s1), PDCL (Mm01327170_m1), GNAT2 (Mm00492394_m1), GNB3 (Mm00516381_m1), GNG8 (Mm00515877_g1) mRNAs were measured on an Applied Biosystems 7500 Real-Time PCR System. The PCR was performed under the following conditions: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. The data were normalized to the Actb reference. The RT-PCR results were analyzed using the ∆∆Ct method to compare the mRNA levels of PhLP1F/FCre+ mice to controls. At least three animals of each genotype were used, and the RT-PCR analysis was repeated three times for each animal.
Assessment of the photo-response by Electroretinography

Electroretinograms (ERG) were measured under photopic conditions, high light intensities that activate cones and inactivate rods, as follows. One-month old mice were first anesthetized with isofluorane and their pupils were dilated by adding a drop of 1% tropicamide for 15 min to the eyes. A recording electrode was placed on the cornea with a reference electrode inserted subdermally in the cheek and a ground electrode subdermally at the base of the tail. ERG responses were measured using an Ocuscience HMsERG system. Mice were first light adapted for 10 min. at light intensity of 30 cd·s m⁻². Full-field photopic ERG recordings of both PhLP1⁺/⁻Cre⁺ and PhLP1⁺/+Cre⁺ mice were performed with flashes of increasing white light intensities from 2.3 ×10⁻² to 1.0 ×10² cd·s m⁻² followed by a recovery phase at 30 cd·s m⁻². The recovery time between each flash varied from 10 s to 2 min depending on the flash intensities. The amplitudes of the photopic b-wave at different light intensities were then compared between the PhLP1⁺/⁻Cre⁺ and PhLP1⁺/+Cre⁺ mice. The intensity–response data were fitted to the Naka–Ruston hyperbolic function with the Hill coefficient set to 1.

Electroretinograms were also measured under scotopic conditions, low light intensities that only activate rods, as follows. One-month old mice were dark adapted overnight and then treated as above without any light adaptation. Full-field scotopic ERG recordings of both PhLP1⁺/⁻Cre⁺ and PhLP1⁺/+Cre⁺ mice were performed with flashes of increasing white light intensities from 2.0 ×10⁻³ to 1.0 ×10² cd·s m⁻². The recovery time of the scotopic ERG between each flash varied from 10 s to 4 min. depending on the flash intensities. The amplitudes of the a-wave and b-wave at different light intensities were then compared between the PhLP1⁺/⁻Cre⁺ and PhLP1⁺/+Cre⁺ mice. The intensity–response data were fitted to a double hyperbolic function (114), again with Hill coefficients set to 1.
**Optomotor responses**

PhLP1\(^{+/−}\)Cre\(^{+}\) mice were bred with GNAT\(^{−/−}\) mice to create a double knock-out PhLP1\(^{+/−}\)Cre\(^{+}\)GNAT\(^{−/−}\) to remove rod signaling that could interfere with optomotor response tests. Visual acuity and contrast sensitivity of PhLP1\(^{+/−}\)Cre\(^{+}\)GNAT\(^{−/−}\) and PhLP1\(^{+/+}\)Cre\(^{+}\)GNAT\(^{−/−}\) mice were measured using a two-alternative forced-choice protocol (115). The Optomotry system (Cerebral Mechanics) consisted of a square array of four computer monitors with a pedestal in the center where the mouse was placed. An infrared-sensitive television camera and a round array of six infrared LEDs mounted above the animal were used to observe the mouse but not the monitors. Using a staircase paradigm, rotating stimuli (sine-wave vertical gratings) were applied on the monitors where they formed a virtual cylinder around the mouse (116). The mouse responded to the stimuli by reflexively rotating its head in the corresponding direction. Optomotor responses were measured under two background illumination conditions: scotopic (−4.45 log cd m\(^{−2}\)) or photopic (1.85 log cd m\(^{−2}\)). For scotopic conditions, the background monitor luminance was controlled by neutral density film filters.

Visual acuity was defined as the threshold for spatial frequency (F\(s\)) of gratings with 100% contrast and measured at the speed (S\(p\)) of 12°/s for both illumination conditions. In this mode, F\(s\) was gradually increased by the computer protocol until its threshold was determined. Temporal frequency (F\(t\)) was automatically adjusted by the computer program, based on the following equation: F\(t\) = S\(p\) × F\(s\) (115). Contrast sensitivity was defined as the inverse of contrast threshold for optomotor responses. In this mode, contrast of the stimuli was gradually decreased by the computer protocol until its threshold was determined. F\(s\) was fixed at 0.128 cycles/degree, F\(t\) was set to 1.5 Hz, and S\(p\) was set to 12°/s for both illumination conditions. Data were
analyzed using independent two-tailed Student's t test, with an accepted significance level of \( p < 0.05 \).

**Transretinal ERG Recordings**

Transretinal ERG recordings were performed as described previously (117). Briefly, four-week-old \( PhLP1^{+/+} Cre^+ GNAT^{+/+} \) and \( PhLP1^{+/+} Cre^+ GNAT^{+/+} \) mice were dark-adapted overnight and then whole retinas were removed from dissected eye cups and prepared for transretinal recordings. Cone-driven test flash responses were recorded at intensities from \( 2.5 \times 10^3 \) to \( 6.0 \times 10^7 \) photons (\( \mu m^2 \)). The transretinal recordings were done using synaptic inhibitors to block post-photoreceptor components of the photoresponse. The intensity–response data were fitted to the Naka–Ruston hyperbolic function.

**Results**

**Confirmation of the cone-PhLP1 deletion**

To assess the role of PhLP1 in the assembly of \( G\beta_3\gamma_8 \) and RGS9-\( G\beta_5 \) in cone photoreceptors, we created a cone-specific knockout of PhLP1 by crossing the \( PhLP1^{loxP} \) (\( PhlpF \)) mouse (70) with the HRGP mouse in which cone expression of Cre recombinase is driven by the human cone red-green opsin promoter (108,118). Cre-mediated recombination causes the loss of the translation initiation site of PhLP1, thus removing PhLP1 from cones as soon as the opsin expression. Full disruption of the \( Phlp1 \) gene was achieved by generating mice that were homozygous for the \( PhlpF \) allele and heterozygous for HRGP-\( cre \) allele. The presence of the \( PhlpF \) gene was confirmed (Figure 2-3A) by a shift in the PCR product (704 bp) compared to the wild type allele (600 bp). PhLP1 protein expression was then tested by immunohistochemistry of PhLP1 in retinal cross-sections. However, it was difficult to distinguish PhLP1 expression in cones from that in rods in the photoreceptor layer. To overcome
this problem, we crossed our PhLP1<sup>F/F</sup>Cre<sup>+</sup> mouse line with a mouse line expressing enhanced green fluorescent protein (EGFP) specifically in cones to create a PhLP1<sup>F/F</sup>Cre<sup>+</sup>EGFP<sup>+</sup> mouse line with an EFGP marker in the cones (104). Immunolocalization of PhLP1 in these mice showed strong PhLP1 staining in the outer segment of cones with the wild type Phlp allele (PhLP1<sup>+/+</sup>Cre<sup>+</sup>EGFP<sup>+</sup>) with noticeably weaker staining in rods. In contrast, PhLP1 staining was essentially absent in cones from the knockout mice while rod staining was unchanged (Figure 2-3B). This result shows that PhLP1 protein expression was specifically lost in the cones of PhLP1<sup>F/F</sup>Cre<sup>+</sup>EGFP<sup>+</sup> animals.

![Figure 2-3. Characterization of the cone photoreceptor-specific PhLP1 knock-out mouse.](image)

A) PCR genotyping results using the PhLP-loxP primers. The PhLP<sup>F</sup> gene generated a 704 bp product, while the wild-type generated a 600 bp product. B) Immunolocalization of PhLP1 (red) in retinal cross-sections from PhLP<sup>+/+</sup>Cre<sup>+</sup> and PhLP<sup>F/F</sup>Cre<sup>+</sup> mice expressing EGFP (green) in cone. An example is indicated by a white arrow. C) Immunoblot detection of PhLP1 from whole-retinal lysates from PhLP<sup>+/+</sup>Cre<sup>+</sup> and PhLP<sup>F/F</sup>Cre<sup>+</sup> mice. D) Immunolocalization of TRITC-labeled PNA (red) in retinal cross-sections from 1-month and 9-month-old PhLP<sup>+/+</sup>Cre<sup>+</sup> and PhLP<sup>F/F</sup>Cre<sup>+</sup> mice.
The greater immuno-staining of PhLP1 in cones than in rods was unexpected because it had not been observed previously (70,109). The difference can be attributed to immunolocalization conditions that have been optimized to specifically detect PhLP1 (see Experimental Procedures). The enhanced PhLP1 detection suggests that PhLP1 expression is much higher in cones than in rods. To further investigate this notion, PhLP1 immunoblots of whole retinal lysates from $PhLP1^{F/F}Cre^+$ and $PhLP1^{+/+}Cre^+$ mice (Figure 2-3C) were compared. There was a 40% decrease in PhLP1 in the knockout, despite the fact that cones only represent ~3% of all photoreceptors suggesting that PhLP1 expression is ~10-fold higher in cones than in rods.

Assessment of cone viability

In rod cells, loss of PhLP1 resulted in measurable degeneration of the photoreceptor layer after one month and nearly complete loss by 6 months (70). This degeneration was evident by shortening of the photoreceptor layer and loss of nuclei. To determine if a similar effect would be seen in cone cells, we stained cones of one month and nine month old mice with a TRITC conjugated peanut agglutinin, which stains the exterior of cone inner and outer segments (119). $PhLP1^{F/F}Cre^+$ and $PhLP1^{+/+}Cre^+$ mice showed similar number and size of cone cells in both one and nine month old animals (Figure 2-3D) indicating that PhLP1 deletion does not cause cone degeneration.

PhLP1 deletion causes a decrease in cone $G_\text{t}$

Although their overall mechanism for G protein signaling is the same, rods and cones have a different $G_\text{t}$ heterotrimeric. Rod photoreceptors use $G\alpha_{t1}$, $G\beta_1$ and $G\gamma_1$, whereas cones use $G\alpha_{t2}$, $G\beta_3$ and $G\gamma_8$. Thus the deletion of PhLP1 in cones allowed an evaluation of the contribution of PhLP1 to $G\beta_3G\gamma_8$ assembly in vivo. We first measured the expression of the cone
$G_t$ subunits in $PhLP1^{+/+} Cre^+$ and $PhLP1^{+/+} Cre^+$ mice by immunohistochemistry. The $PhLP1^{FF} Cre^+$ mice showed a marked decrease in immunolabeling of $G_{αt2}$, $Gβ_3$ and $Gγ_8$ in the cones (Figure 2-4A), indicating that expression of the cone $G_t$ subunits was substantially reduced. In addition, the residual $Gαt2$ was mis-localized in the absence of PhLP1, with more staining in the cell body and less staining in the outer segment. The effect appeared specific for the cone $G_t$ subunits because there was no difference in cone opsin expression or localization.

To further assess the effects of PhLP1 deletion on cone $G_t$ expression, whole retina extracts were immunoblotted for cone $G_t$ subunits, other cone proteins and rod $G_t$ subunits. $Gαt2$ and $Gγ_8$ were both reduced significantly in the PhLP1 knockout, while $Gβ_3$ was not (Figure 2-5A

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**Figure 2-4. Immunolocalization of G-protein subunits in PhLP-deleted cones.** A) Retinal cross-sections from $PhLP^{+/+} Cre^+$ and $PhLP^{FF} Cre^+$ mice were probed with antibodies specific to $Gαt2$, $Gβ_3$, $Gγ_8$, and cone opsin and detected with FITC-conjugated secondary antibodies. B) Retinal cross-sections from $PhLP^{+/+} Cre^+$ and $PhLP^{FF} Cre^+$ mice expressing EGFP in cones were probed with antibodies specific to RGS9-1 and $Gβ_3$ and detected with AF555-conjugated secondary antibodies. Orange regions of overlapping fluorescence indicate expression of the probed proteins in cones. Examples are indicated by white arrows.
and B). The lack of change in $G\beta_3$ expression in whole retina was not surprising since the PhLP1 deletion was limited to cone cells and $G\beta_3$ is also expressed in other retinal cells (120,121). The expression of other cone proteins, opsin and arrestin, was unchanged in the absence of PhLP1 (Figure 2-5B), indicating that the loss of PhLP1 specifically affected cone $G\zeta$ subunit expression and not cone protein expression in general. Rod $G\zeta$ subunit expression was also unchanged, demonstrating that the effect was limited to cones.

To determine if the reduction in cone $G\zeta$ subunit expression were caused by transcriptional or post-transcriptional events, we measured the effects of PhLP1 depletion on the cone $G\zeta$ subunit mRNA levels in whole retinal extracts. No differences in $G\alpha_{12}$, $G\beta_3$ or $G\gamma_8$ mRNA levels were observed (Figure 2-5C), indicating that the reduced expression was caused by post-transcriptional events.

PhLP1 deletion causes a decrease in cone RGS9-$G\beta_5$

We previously observed that PhLP1 deletion in rods caused a striking >95% decrease in RGS9-$G\beta_5$ expression in those cells, most likely because of an inability to form RGS9-$G\beta_5$ dimers (70). The cone-specific PhLP1 deletion provided an opportunity to test whether this strict PhLP1-dependence for RGS9-$G\beta_5$ assembly seen in rods was also the case in cones. To address this question, we measured the effect of PhLP1 deletion on $G\beta_5$ and RGS9 expression in cones by immunohistochemistry. We again used EGFP expressing cones to distinguish between cone and rod expression because the extensive $G\beta_5$ and RGS9 expression in rods can mask changes in their expression in cones. In the $PhLP1^{+/+} Cre^+ EGFP^+$ wild-type mice, expression of RGS9 was clearly observed in cone outer segments as evidenced by the orange color produced by overlapping fluorescence signals from EGFP (green) and RGS9 (red) in the merged image (Figure 2-4B). In contrast, the $PhLP1^{+/-} Cre^+ EGFP^+$ knockout mice showed no orange cone outer
segments in the merged image, indicating that RGS9 expression was substantially decreased in PhLP1-depleted cones.

We applied the same strategy to assess Gβ5 expression in cones and observed a similar result (Figure 2-4B). The number of orange cone outer segments indicating expression of Gβ5 in cones was much less in the PhLP1-deleted cones than in the wild-type cones (Figure 2-4B). This decrease in both RGS9 and Gβ5 expression in PhLP1-deleted cones argues that RGS9-Gβ5 dimer

![Figure 2-5. Protein Expression in PhLP-deleted cones. A) Immunoblots of whole-retinal extracts for PhLP1, G12 subunits, RGS9-1, Gβ5, opsin, cone arrestin, and G7 subunits. B) Quantification of the immunoblot bands in A relative to the wild-type. C) Levels of indicated mRNAs in whole-retinal extracts were determined by RT-PCR.](image)
formation is as dependent on PhLP1 in cones as it is in rods. We attempted to confirm the decreased expression by immunoblotting whole retinal extract for Gβ5 and RGS9, but saw no differences (Figure 2-5B) most likely because the high level of Gβ5 and RGS9 expression in rods negates the ability to detect changes in cones.

**PhLP1 knockout results in decreased photopic phototransduction**

The loss of cone G protein and RGS protein expression should have a profound effect on phototransduction in cones. To test this possibility, we performed a full-field ERG analysis on PhLP1F/FCre+ and PhLP1+/+Cre+ mice. Photopic ERG responses, which rely on cone vision in bright light, were desensitized in PhLP1F/FCre+ mice compared to control mice, as evidenced by the decreased amplitude of the b-wave to a series of increasing light flashes (Figure 2-6A). The stimulus-response curve showed a nearly 10-fold decrease in sensitivity, as evidenced by the increased light-intensity required to produce a half-maximal response (I1/2) in the PhLP1-deleted animals, (Figure 2-6C and Table 2-1). Despite the decrease in light sensitivity, there was no significant change in the maximal response amplitude (Rmax) at high light intensity (Figure 2-6C and Table 2-1). In contrast, scotopic ERG responses, which stem from rod vision under dark-adapted conditions, were essentially the same in the PhLP1F/FCre+ and PhLP1+/+Cre+ mice (Figure 2-6B). The sensitivity and amplitude of both the scotopic a-wave and b-wave were not significantly different in the knockout mice (Figures 2-6D and E), indicating that rod phototransduction was unaffected by the cone PhLP1-deletion. These ERG results demonstrate that cone vision is severely impaired in cone-specific PhLP1 knockout mice, as would be expected from the loss cone Gt and RGS9-Gβ5 complexes.

To clearly isolate cone photoresponse from those of rods, we bred the PhLP1F/FCre+ line onto a Gα1 knockout background (GNAT−/−), which removes the Gα1 subunit from rod cells and
thus eliminates rod signaling (103). These mice were first tested for visual acuity and contrast sensitivity by optomotor responses to rotating grid stimuli (70). We found that PhLP1F/F Cre+GNAT−/− mice showed a 35% reduction in visual acuity compared to PhLP1+/+Cre+GNAT−/− animals (Figure 2-7A).

Figure 2-6. ERG analysis of cone-specific PhLP1-depleted mice. A-B) Families of ERG responses for PhLP1+/− Cre+ and PhLP1F/F Cre+ mice for photopic (A) and scotopic (B) tests. Light intensity values are in log candela seconds per square meter. C) Intensity-response relationships for photopic b waves. Data were fit to the Naka-Ruston function that yielded the parameters in Table 2-1. D-E) Intensity-response relationships for scotopic a waves (D) and scotopic b waves (E). Data were fit to a double hyperbolic function (114).

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animals showed greater impairment with a nearly 14-fold reduction compared to wild-type (Figure 2-7B). These results further demonstrate that cone vision is significantly diminished in PhLP1-deleted cones.

To investigate the effects of PhLP1 deletion on cone signaling more specifically, we measured photoresponses by transretinal recordings of dark-adapted mice retinas with the GNAT\textsuperscript{\textminus/} background at increasing light intensities. Recordings were done using synaptic inhibitors to block post-photoreceptor components of the photoresponse. Similar to full-field ERG readings, transretinal recordings from PhLP1\textsuperscript{F/F\textasciicircum{C}re\textsuperscript{+}GNAT\textsuperscript{\textminus/}} mice showed substantial desensitization compared to wild-type controls (Figure 2-7 C, D). The decreased sensitivity could be easily seen by comparing the responses at 5.7×10\textsuperscript{4} photons \(\mu\text{m}^{-2}\) (Figure 2-7C & D: red lines). Stimulus-response curves further illustrated the decreased sensitivity, showing a 58-fold increase in I\textsubscript{1/2} in the knockout mice with no change in R\textsubscript{max} (Figure 2-7E and Table 2-2). This desensitization is nearly six times larger than that seen in the full-field ERG recordings, suggesting that rod photoresponses were not completely abrogated in the full-field ERG tests. Both the photopic ERGs and the transretinal recordings showed no difference in the maximum cone response amplitudes between PhLP1\textsuperscript{+/C\textasciicircum{re\textsuperscript{+}} and PhLP1\textsuperscript{F/F\textasciicircum{C\textasciicircum{re\textsuperscript{+}} mice (Figure 2-7E), indicating that the number and length of the cones were the same in the two mouse lines as observed in the cone morphology data (Figure 2-3D). From the trans-retinal data, we were able to assess the effect of PhLP1 deletion on the cone photoresponse amplification rate by comparing the intensities of light require to produce identical response activation phases. We compared population-averaged fractional responses in the linear range that corresponded to 5.7×10\textsuperscript{4} photons \(\mu\text{m}^{-2}\) for PhLP1\textsuperscript{F/F\textasciicircum{C\textasciicircum{re\textsuperscript{+} GNAT\textsuperscript{\textminus/}} retina, and

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<th>Table 2-1. ERG intensity Parameters for Photopic b-waves.</th>
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<td>(I_{1/2}(\text{cd}\cdot\text{s}\cdot\text{m}^{-2}))</td>
<td>(R_{\text{max}}(\mu\text{V}))</td>
<td></td>
</tr>
<tr>
<td>PhLP1\textsuperscript{+/C\textasciicircum{re\textsuperscript{+}} (n=8)</td>
<td>4.58 ± 0.39</td>
<td>180 ± 3</td>
</tr>
<tr>
<td>PhLP1\textsuperscript{F/F\textasciicircum{C\textasciicircum{re\textsuperscript{+}} (n=8)</td>
<td>39.05±11.42</td>
<td>168 ± 19</td>
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Figure 2-7. Visual behavioral and transretinal ERG responses of PhLP1-deleted mice. A) Photopic visual acuity. B) Photopic contrast sensitivity. All data are means ± SEM. C-D) Representative families of transretinal ERG flash responses from PhLP<sup>+/−</sup> Cre<sup>−</sup> GNAT<sup>−/−</sup> (C) and PhLP<sup>1F/FCre+GNAT−/−</sup> (D) mice are shown. Test flashes of increasing intensities were delivered at time 0. The red traces show responses to an intensity of 5.7×10<sup>4</sup> photons µm<sup>−2</sup>. E) Intensity response functions for transretinal ERG responses from PhLP<sup>+/−</sup> Cre<sup>−</sup> GNAT<sup>−/−</sup> (n=9) and PhLP<sup>1F/FCre+GNAT−/−</sup> (n=13) mice. Data were fit to the Naka-Ruston function that yielded the parameters in Table 2-2. F) Amplification of the phototransduction cascade in mouse cones. The inset shows the rising phase of the responses on an extended time scale. See text for details. G) Kinetics of the dim flash responses. Normalized population-averaged dim flash responses to light intensities of 2.4×10<sup>3</sup> photons µm<sup>−2</sup> for PhLP<sup>1+/−</sup> Cre<sup>−</sup> GNAT<sup>−/−</sup> (n=9) and 5.7×10<sup>4</sup> photons µm<sup>−2</sup> for PhLP<sup>1+/−</sup> Cre<sup>−</sup> GNAT<sup>−/−</sup> (n=12), demonstrating the decelerated photoresponse recovery in PhLP1-deleted cones. The inset shows an extended time scale.
2.4×10³ photons µm⁻² for PhLP1+/+Cre+GNAT⁻/⁻ retina. To obtain the best match of the rising phase, the fractional dim flash PhLP1⁺/FCre⁺GNAT⁺⁻ response required downscaling by a factor of 4.5. The ratio of the two light intensities corrected by the scaling factor yielded a 5.2-fold reduction in the signal amplification rate in PhLP1⁺/FCre⁺GNAT⁻/⁻ retina. This reduction corresponds well to the nearly 3.5-fold reduction seen in Ga2 levels observed in PhLP1⁺/FCre⁺GNAT⁻/⁻ cones.

**PhLP1 knockout results in prolonged photo response recovery**

RGS9-Gß₅ is highly expressed in cones and is believed to contribute substantially to the rapid photoresponse recovery rate characteristic of cones (107,122,123). Thus, the loss of RGS9-Gß₅ upon PhLP1 deletion (Figure 2-4B) would be expected to decrease the recovery rate. In fact, there was a striking delay in the recovery phase of the cone photoresponse (Figure 2-7G). The dim flash recovery time constant (τrec) was increased 50-fold (Table 2-2), ten times more than was seen upon PhLP1 deletion in rods (70). This dramatic decrease in the cone recovery rate is very similar to that observed in RGS9 knockout mice (123) and provides addition evidence that RGS9-Gß₅ plays a key role in the rapid kinetics of cone photoresponses.

**Discussion**

**PhLP1 and cone Gt function**

Specific deletion of PhLP1 in cone photoreceptors demonstrates its essential role in cone physiology. The loss of PhLP1 substantially reduced expression of the subunits of the cone Gt heterotrimer (Figures 2-4 and 2-5), and resulted in a marked attenuation of photopic photoresponses (Figures 2-6 and 2-7). These findings mirror those of the rod-specific PhLP1

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<th>Table 2-2. Transretinal ERG parameters.</th>
<th>( I_{1/2} ) (photons um⁻²)</th>
<th>( R_{max} ) (µV)</th>
<th>( \tau_{rec} ) (ms)</th>
<th>( T_{peak} ) (ms)</th>
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<tr>
<td>PhLP1⁺/⁺Cre⁺ (n=9)</td>
<td>1.5×10⁴ ± 1.1×10⁴</td>
<td>51.9 ± 0.95</td>
<td>22</td>
<td>56</td>
</tr>
<tr>
<td>PhLP1⁺/⁺Cre⁺ (n=13)</td>
<td>8.6×10⁵ ± 3.5×10⁵</td>
<td>52.0 ± 5.92</td>
<td>1110</td>
<td>226</td>
</tr>
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deletion, which showed similar reductions in rod Gt subunits, resulting from an inability to form Gβ1γ1 heterodimers (70). Likewise, the observed loss of cone Gt can be attributed to an inability to form Gβ3γ8 dimers in the absence of PhLP1. This observation provides in vivo evidence for the hypothesis, developed from studies in cell culture, that all Gβγ dimer combinations require PhLP1 for their assembly (95). The loss of Gβ3γ8 leads to a reduction in Gαt2 and its mis-localization from the cone outer segment to the inner segment and cell body (Figure 2-4A).

Interestingly, a recent study of a Gβ3 knockout mouse showed a similar mis-localization of Gαt2 in the outer segment (124), lending further support to the idea that Gβ3γ8 plays an important role in the localization of Gαt2 to the outer segment.

The reduction in cone Gt subunits was accompanied by a substantial decrease in photopic vision, demonstrated by full-field ERG and visual behavioral tests (Figure 2-6 and 2-7A, B). This effect was even greater in trans-retinal ERG tests, with a 58-fold decrease in cone light sensitivity and a 5.2-fold decrease in the amplification constant, when all rod signaling was removed by Gαt1 deletion (Figure 2-7, Table 2-2). However, the maximum amplitude of the cone photoresponse was unchanged, indicating that other components of the cone visual cascade such as cone opsin, phosphodiesterase and the cGMP-gated cation channel remained intact in the PhLP1 knockout. These effects on cone phototransduction are similar to those of the Gβ3 knockout (124), supporting the idea that formation of functional Gβ3γ8 dimers was greatly reduced in the absence of PhLP1.

The residual cone photoresponse in the absence of PhLP1 or Gβ3 raises questions about the role of Gβ3γ8 in Gαt2 activation by cone opsin. In both the Gβ3 knockout and the cone PhLP1 knockout, the decrease in the amplification constant was roughly proportional to the decrease in Gαt2 levels in the cone outer segment. This observation suggests that Gβ3γ8 does little to improve
the efficiency of Gα₂ coupling to cone opsin. Considerable insight into G protein activation has come from the atomic structure of the complex between Gs and the agonist-bound β-adrenergic receptor (24). In this complex, there were no direct contacts between Gβ₁γ₂ and the receptor, but interactions between Gβ₁ and the N-terminus of Ga₅ positioned the N-terminus next to the membrane where it made important contacts with the receptor. In the case of Gt₂ and cone opsin, these interactions may be less important for activation. It should be noted that residual signaling probably does not result from compensation by another Gβγ pair in Gα₂ activation because in the PhLP1 knockout all Gβγ assembly should be equally disrupted in the absence of PhLP1 (95).

Collectively, these observations suggest that the most important role of Gβ₃γ₈ in cone signaling is not to improve receptor coupling, but to stabilize Gα₂ and increase its interaction with the cone outer segment membrane.

PhLP1 and RGS9-Gβ₅ assembly in cones

The loss of Gβ₅ and RGS9 in the cone PhLP1 knockout (Figure 2-4B) is indicative of an inability to form RGS9-Gβ₅ dimers. Previous work has shown that deletion of either Gβ₅ or RGS9 results in complete loss of the other (113,125). This profound loss of both Gβ₅ and RGS9 in the absence of PhLP1 was also observed in rods (70), thus both rods and cones exhibit a strict requirement for PhLP1 in RGS9-Gβ₅ assembly. Several studies have shown that cones express higher levels of RGS9-1 and Gβ₅ than rods, which is believed to contribute to the rapid deactivation kinetics of cones (107,122). Perhaps this is why PhLP1 expression was found to be higher in cones than in rods (Figures 2-3 and 2-5).

The loss of RGS9-Gβ₅ complexes resulted in a 50-fold increase in recovery time (Figure 2-7, Table 2-2). These results parallel those measured in cones of RGS9⁻/⁻ mice, which showed a 60-fold increase in recovery time (123). This nearly identical increase suggests that RGS9-Gβ₅
complexes are virtually non-existent in PhLP-deleted cones, advocating a strict dependence of RGS9-Gβ5 complex formation on PhLP-mediated assembly.

**PhLP1 and cone viability**

PhLP1 deletion in rods resulted in photoreceptor degeneration, yet we did not observe a similar degeneration in cones (Figure 2-3D). The proposed cause of rod degeneration was the accumulation of Gβ1 on CCT (70). The lack of degeneration in cones would suggest that the chaperonin system is not as compromised by the loss of PhLP1. One possibility is that Gβ3 is more easily cleared from CCT than Gβ1. Consistent with this hypothesis, Gβ3 has been shown to have lower affinity for CCT than Gβs 1, 2, and 4 (68). Alternatively, rod-derived survival factors may maintain cone viability (126,127) despite possible insults to the cone proteome from diminished CCT function in the absence of PhLP1.

In summary, the deletion of PhLP1 in cone photoreceptors results in the loss of cone Gt heterotrimers and RGS9-Gβ5 dimers and leads to a marked reduction in cone light sensitivity and a greatly retarded photoresponse recovery. These findings mimic those of the PhLP1 deletion in rod photoreceptors (70), demonstrating a common mechanism of Gβγ and RGS9-Gβ5 in rods and cones. These results predict that PhLP1 and CCT-mediated assembly of these complexes is shared in other neurons, highlighting the importance of these chaperones in G protein signaling.
CHAPTER 3:
PROGRAMMED CELL DEATH PROTEIN 5 INTERACTS WITH THE CYTOSOLIC CHAPERONIN CCT TO REGULATE β-TUBULIN FOLDING

Summary

Programmed cell death protein 5 (PDCD5) has been proposed to act as a pro-apoptotic factor and tumor suppressor. However, the mechanisms underlying its apoptotic function are largely unknown. A proteomics search for binding partners of phosducin-like protein, a co-chaperone for the chaperonin CCT, revealed a robust interaction between PDCD5 and CCT. PDCD5 forms a complex with CCT and β-tubulin, a key CCT folding substrate, and specifically inhibited β-tubulin folding. Cryo-electron microscopy studies of the PDCD5-CCT complex suggested a possible mechanism of inhibition of β-tubulin folding. PDCD5 bound the apical domain of the CCTβ subunit, projecting above the folding cavity without entering it. Like PDCD5, β-tubulin also interacts with the CCTβ apical domain, but a second site is found at the sensor loop deep within the folding cavity. These orientations of PDCD5 and β-tubulin suggest that PDCD5 sterically interferes with β-tubulin binding to the CCTβ apical domain and inhibits β-tubulin folding. Given the importance of tubulins in cell division and proliferation, PDCD5 might exert its apoptotic function at least in part through inhibition of β-tubulin folding.

Introduction

In seeking to determine if PhLP1 were involved in the folding of other CCT substrates, particularly other β-propeller proteins, we identified a novel interaction of CCT with

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*This chapter also represents the work of several people including Aly Howlett, Amy Gray, John Prince, Tanner Shaw, and Rebecca Plimpton of BYU and Jorge Cuellar of the Valpuesta Lab at the Centro Nacional de Biotechnologia, Madrid, Spain. I specifically performed the work represented in figures 3-1D, 3-3 B-F, 3-6C, 3-7, and 3-8 and Table 3-2.
programmed cell death protein 5 (PDCD5). PDCD5 was originally described as an up-regulated gene from cells undergoing apoptosis (129). Cellular expression of PDCD5 is decreased in many cancer cell lines and tumors (130,131). Moreover, over-expression of PDCD5 accelerates apoptosis in tumor cells (132), suggesting that PDCD5 plays a role in stemming uncontrolled cell proliferation by triggering apoptosis. It has been proposed that apoptotic stimuli cause translocation of PDCD5 to the nucleus, where it interacts with the histone acetyltransferase Tip60 and the transcription factor p53 to promote programmed cell death (133,134).

In contrast to these observations, our data show that PDCD5 interacts with CCT in the cytosol as a regulatory co-chaperone that specifically inhibits β-tubulin folding. Based on these findings, we propose that the apoptotic activity of PDCD5 may result at least in part by impairing CCT-mediated β-tubulin folding.

**Experimental Procedures**

*Cell culture*

HEK-293T and U2OS cells were cultured in 1:1 DMEM/F-12 growth media containing 2.5 mM L-glutamine and 15 mM HEPES supplemented with 10% fetal bovine serum. The cells were sub-cultured regularly to maintain growth but were not used beyond 20 passages.

*Preparation of cDNA constructs*

Human PDCD5 (N-terminal FLAG tagged, N-terminal FLAG-TEV tagged, or C-terminal FLAG tagged) and tubulin-binding protein co-factors A (TBCA) and B (TBCB) with C-terminal FLAG tags (Open Biosystems) were cloned in the pcDNA3.1/myc-His B vector (Invitrogen) using PCR. C-terminally e-myc-tagged human PhLP1 in pcDNA3.1/myc-His B vector was described previously (64). For recombinant protein purification, His<sub>6</sub>-PDCD5-FLAG was cloned into the first multiple cloning site of the bacterial expression vector pETDuet, and PhLP1-myc-
His was cloned into the bacterial expression vector pET15b (Novagen) using PCR. The integrity of all constructs was confirmed by sequence analysis. The N-terminally hemagglutinin (HA)-tagged Gγ2 and FLAG epitope-tagged Gβ1 cDNAs also in the pcDNA3.1 vector were obtained from the UMR cDNA Resource Center. BzF tRNA and synthetase cDNAs were a generous gift from Thomas Sakmar (Rockefeller University).

*Transient transfections*

HEK-293T and U2OS cells were grown in 6-well plates, 60-mm dishes, or 100-mm dishes to 80-90% confluency at which point they were transfected with 1 μg (6-well plate), 2-5 μg (60-mm dishes), or 6 μg (100-mm dishes) each of the indicated vectors using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). After 48 hours, the cells were harvested for immunoprecipitation, mass spectrometry, or radiolabel pulse-chase experiments.

*Protein Expression and Purification*

*Escherichia coli* DE3 cells were transformed with human PhLP1 in the pET15b vector or PDCD5 in the pETDuet vector. The recombinant proteins were then purified using Co²⁺ affinity chromatography as previously described for Ni²⁺-chelate chromatography (79). The purified proteins were concentrated and exchanged into 20 mM HEPES pH 7.2, 150 mM NaCl by ultrafiltration and were stored in 40% glycerol at −20°C. Protein concentrations were determined using BCA protein assay reagent (Pierce). CCT was purified from bovine testis as described previously (14).

*Immunoprecipitation experiments*

Transfected or untreated HEK-293T or U2OS cells were washed with phosphate-buffered saline (PBS) and solubilized in one of the following immunoprecipitation (IP) buffers depending on the experiment: standard IP buffer (PBS pH 7.4, 1% NP-40 (Sigma)), β-tubulin IP buffer (50
mM Na$_2$HPO$_4$ pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1 mM GTP), β-actin IP buffer (20 mM HEPES pH 7.4, 50 mM KCl, 1 mM DTT, 0.2 mM CaCl$_2$, 0.5% NP-40, 4 μM cyclohexamide, 40 mM glucose), or ATP-depletion buffer (PBS pH 7.4, 1% NP-40, 100 mM deoxy-glucose, 1 mM azide, 5 mM EDTA). All were supplemented with 0.6 mM PMSF and 6 μl/ml protease inhibitor cocktail (Sigma P8340). The lysates were passed through a 25-gauge needle 10 times and centrifuged at maximum speed for 10 minutes at 4°C in an Eppendorf microfuge. The protein concentration for each sample was determined using the DC Protein Assay Kit II (Bio-Rad), and equal amounts of protein were used in the subsequent immunoprecipitations. The clarified lysates were incubated for 30 minutes at 4°C with one of the following antibodies as indicated: 3-5 μg anti-myc (clone 9E10, Enzo Life Sciences), 3-8 μg anti-CCTε antibody (clone PK/29/23/8d, AbD Serotec), 3-6 μg anti-FLAG (clone M2, Sigma), or 0.4 μg anti-HA (clone 3F10, Roche). Next, 30 μl of Protein A/G Plus agarose slurry (Santa Cruz Biotechnology) were added, and the mixture was incubated for 20-30 minutes at 4°C. In β-actin precipitations, DNase I agarose beads were used to precipitate folded actin as described previously (57). Immunoprecipitated proteins and lysates were resolved on 10% or 14% Tris-glycine-SDS gels or 16.5% Tricine-SDS gels. The proteins were transferred to nitrocellulose and immunoblotted using the following antibodies as indicated: anti-CCTα, β, δ, ε, η, θ (AbD Serotec), anti-CCTζ (Santa Cruz), anti-CCTγ, α-tubulin, β-tubulin, PDCD5 (Abcam), anti-FLAG or anti-myc antibodies. Immunoblots were incubated with the appropriate anti-mouse, anti-rat, anti-rabbit, or anti-goat secondary antibody conjugated to an infrared dye (LI-COR Biosciences). Blots were scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences), and protein band intensities were quantified using the Odyssey software. In all cases, the ratio of the co-
immunoprecipitated protein to the immunoprecipitated protein was calculated and then normalizing to the control.

**RNA interference experiments**

HEK-293T cells were grown in 12-well or 6-well plates to 40–50% confluency at which point they were transfected with CCTζ (Dharmacon), PDCD5 (Ambion), or negative control #1 (Ambion) siRNA using Oligofectamine (Invitrogen) reagent as described previously (64) or RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol. In some cases, cells were transfected 24 h later with 0.5 μg (12-well) or 1.0 μg (6-well) of the indicated cDNAs using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Cells were harvested for subsequent immunoprecipitation experiments 4 days after the knockdown. A total of 10 μg of cell lysates were immunoblotted with anti-CCTζ or anti-PDCD5 antibodies to assess the percent knockdown.

**Mass spectrometry**

PhLP1 and phosducin (Pdc) binding partners were determined by transfecting their cDNAs with C-terminal tags containing a tobacco etch virus (TEV) cleavage site followed by a myc epitope site (PhLP1-TEV-myc). PDCD5 binding partners were identified by transfecting U2OS cells with an N-terminally tagged FLAG-TEV-PDCD5. Empty vector transfected cells served as a control. After 48 hours, cells were harvested and the lysates (1 mg total protein) were immunoprecipitated with the indicated antibodies. The proteins were released from the antibody-bead complex via TEV protease cleavage according to the manufacturer’s protocol (Promega). The released co-immunoprecipitates were reduced with DTT, alkylated with iodoacetamide and digested with trypsin as described previously (135). Proteins in the co-immunoprecipitates were identified by tandem mass spectrometry (MS/MS). When different, details for the PhLP1 and
Pdc MS/MS analysis are indicated first followed by those from the PDCD5 analysis. MS/MS was performed using an LTQ-Orbitrap mass spectrometer interfaced with a Waters nanoAcquity UPLC, outfitted with a BEH C18 reversed phase column (25 cm x 75 μm i.d., 1.7 μm, 100 or 130 Å, Waters). Peptide mixtures were separated by acetonitrile gradients for 90 min or 150 min at flow rates of 300 or 325 nL/min. MS/MS were collected with m/z window = 475–1600 or 300–2000 Da enabling monoisotopic precursor and charge selection settings. Ions with unassigned charge state or charge state of 1 were excluded. For each MS scan the 5 or 6 most intense ions were targeted with a dynamic exclusion of 30 s, a 20 or 10 ppm exclusion width and a repeat count of 2 or 1. The maximum injection time for Orbitrap parent scans was 500 ms with 1 microscan and automatic gain control of 1x10⁶. The maximum injection time for the LTQ MS/MS was 250 ms with 1 microscan and automatic gain control of 1x10⁴ or 3x10⁴. The normalized collision energy was 35%, with activation Q of 0.25 for 30 ms. Raw files were searched against the UniprotKB human database (including variants) with Sequest, SequestHT, and Mascot (version 2.3) using Proteome Discoverer 1.4. Database search engine parameters were: trypsin digestion, 2 missed cleavages, b and y ion series, precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.8 Da (both assuming monoisotopic peaks), and variable cysteine carboxamidomethylation and methionine oxidation. Additional processing was performed using mspire v0.8.6.2-1-g85f741e(136). Data were transformed to a raw list of spectral counts and filtered to only accept those with Q-value of less than 0.01 (false discovery rate of less than 1%) and protein sequences were inferred using peptide_hit_qvalues_to_spectral_counts_table.rb from mspire which uses QSpec (version 2) (137) with the normalize flag, which normalizes total spectral counts per sample. Table 2 shows average QSpec normalized spectral counts, while Table 1 shows the sum from two biological replicates without QSpec.
normalization. A separate PhLP1 vs. control analysis was performed using QSpec so that a reasonable significance value could be provided in Table 1.

Radiolabel pulse-chase assays

For the rate of CCT association, HEK-293T cells in 6 well plates were transfected with PDCD5-FLAG. After 48 hours, cells were washed and incubated in methionine-free DMEM media (Mediatech Inc.) supplemented with 4 mM L-glutamine (Sigma), 0.063 g/l L-cystine dihydrochloride (USB) and 10% dialyzed fetal bovine serum (Invitrogen) for 1 hr. The media was discarded and the cells were pulsed with new media supplemented with 200 μCi/ml radiolabeled L-[³⁵S] methionine (Perkin-Elmer) for 10 min. The cells were washed and incubated in DMEM/F-12 growth media supplemented with an extra 10 mM L-methionine (Sigma) to stop [³⁵S] methionine incorporation. At increasing times, CCT was immunoprecipitated and the proteins were resolved on 10% Tris-glycine-SDS or 16.5% Tricine-SDS gels. The radiolabeled gels were dried, exposed on a phosphor screen (GE Healthcare) and imaged on a Storm 860 phosphorimager. The band intensities were quantified using Image Quant software (GE Healthcare) and corrected for the number of methionine residues found in each protein. The molar ratios of nascent proteins to CCT ε were then calculated. The rate data were fit to a first-order rate equation to determine the rate constant (k), and the t₁/₂ for assembly or dissociation was calculated as t₁/₂ = ln 2/k.

For protein folding experiments, HEK-293T cells in 12-well plates were treated with PDCD5 siRNA and then transfected 24 hrs. later with FLAG-cofactor B (for α-tubulin folding), FLAG-cofactor A (for β-tubulin folding), FLAG-Gβ₁ and HA-Gγ₂ (for Gβ folding), or nothing (for β-actin folding). At 96 hrs, cells were treated with [³⁵S] methionine as described above and chassed for 60 min (α and β-tubulin), 15 min (β-actin) or 30 min (Gβ). Folded protein was then
determined by the amount of nascent, labeled protein co-immunoprecipitating with its binding partner. Binding partners, co-factor B (TBCB) for α-tubulin, co-factor A (TBCA) for β-tubulin, Gγ for Gβ and DNase I beads for β-actin, associate with their targets immediately after folding by CCT. Radioactive bands were detected and the ratios of the folded protein to its binding partner were calculated and normalized to the control siRNA.

**EM and image processing**

PDCD5-CCT complexes were prepared by mixing purified components in a 10:1 PDCD5 to CCT ratio. For the two-dimensional EM analysis, aliquots of the different samples (CCT, PDCD5-CCT complex, or the immunocomplex between PDCD5, CCT and a monoclonal antibody against CCTδ (PK/9/86b from Genway)) were applied onto carbon-coated copper grids and stained with 2% uranyl acetate. Micrographs were taken under minimal dose conditions in a JEOL JEM1200EXII microscope operated at 100 kV and digitized in a Zeiss SCAI scanner with a sampling window corresponding to 3.5 Å/pixel. Individual particles were manually selected using XMIPP (138). Image classification was performed using a free-pattern maximum-likelihood multi-reference refinement (ML2D) (139). Homogeneous populations were obtained and averaged for a final two-dimensional characterization.

For the three-dimensional reconstruction of the PDCD5-CCT complex, aliquots of the solution were applied to Quantifoil 2-μm holey carbon grids for 1 min, blotted for 3 s and frozen rapidly in liquid ethane at −180 °C. Images were acquired with a defocus range of 2–3.5 μm at 1.75 Å per pixel sampling rate on a 4K x 4K Eagle CCD camera (Gatan Inc.) mounted on a FEI Tecnai G² FEG200 electron microscope at 200 kV with a Gatan side-entry cryo-holder. A total of 13,100 particles (down-sampled to 3.5 Å per pixel) were selected, normalized and CTF-corrected using standard XMIPP procedures (138). Images were classified using ML2D, and the
most representative classes were used to generate an initial 3D model using the startcsym program from the EMAN package (140). The resulting model was subsequently refined without imposing any symmetry. The model was filtered at 50 Å and used for 3D maximum-likelihood classification (ML3D) with internal correction for normalization errors (141) to separate PDCD5-bound CCT particles from the PDCD5-free ones. The selected particles with bound PDCD5 were refined with EMAN to obtain the final model. The resolution of the reconstructions was determined to be 25 Å by the Fourier shell correlation coefficient (FSC) 0.5 criterion between two independent reconstructions (Figure 3-5). The density maps and atomic structures were visualized with UCSF Chimera (142). The atomic structures were manually fitted into the 3D reconstructions.

Amino acid incorporation and photo cross-linking

Amber codon suppression technology was used to incorporate the photo-crosslinking unnatural amino acid p-benzoyl-L-phenylalanine (BzF) into residues at the C-terminus of PDCD5 as described previously (143). PDCD5 variants were engineered with amber codons inserted at residues 111, 113, 115, 117, or 119 of the PDCD5 C-terminus. Five μg of each variant cDNA were transfected into HEK-293T cells plated on 60 mm dishes using Lipofectamine 2000, along with 5 μg of BzF tRNA and 0.5 μg of BzF tRNA synthetase cDNA. Three hours post-transfection, the cell media was supplemented with fresh media containing BzF, bringing the final concentration to 1 mM. Cells were then harvested in IP buffer at 40-48 hours post-transfection and clarified lysates were exposed to UV light for 4 minutes at 4°C using a 600 W UV lamp (150 mW/cm², Integrated Dispensing Solutions) set to half intensity at a distance of 27 cm. FLAG-PDCD5 variants or CCTε were immunoprecipitated and immunoprecipitates and lysates were analyzed by immunoblotting. To test the effects of BzF alone on cross-linking,
similar experiments were performed, but without transfecting the BzF tRNA or synthetase. BzF (1 mM) was added to the cell culture media as indicated.

**Results**

*PDCD5 forms a ternary complex with PhLP1 and CCT*

To investigate possible contributions of PhLP1 to the folding of other proteins besides Gβ, we performed an extensive proteomic analysis of PhLP1 binding partners (Table 1). Immunoprecipitates of PhLP1 were analyzed for potential interactors by tryptic digestion and mass spectrometric identification of the resulting peptides. Multiple peptides from all of the CCT subunits were found in the PhLP1 sample along with Gβ1, Gβ2 and Gβ4 as expected. A phosducin (Pdc) immunoprecipitate was also analyzed for comparison. The Pdc sample contained peptides from the same Gβ subunits but none of the CCT subunits. These results are

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<th>Gene</th>
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HEK293T cells were transfected with PhLP-TEV-myc or Pdc-TEV-myc for comparison. An empty vector transfection served as a negative control. Myc immunoprecipitates were analyzed for binding partners by tandem mass spectrometry. The table displays significant hits found in the proteomics screen. The numbers in the first three columns indicate the normalized spectral counts for each sample. Values in the fourth column indicate the significance of the peptide hits (*Decibans calculated from Bayes factors taken from separately run Qspec analysis of PhLP1 vs. Control).
consistent with previous findings that showed an interaction between PhLP1 and CCT that was not shared with Pdc (57). Interestingly, several peptides from PDCD5 and the peroxisomal multifunctional enzyme type 2 (HSD17B4) were also found in the PhLP1 sample, suggesting an interaction between PhLP1 and these proteins. The apparent interaction between PhLP1 and PDCD5 was intriguing given the proposed tumor suppressor role of PDCD5 (132), so we decided to explore it further. To determine the specificity of the interaction, several PhLP1 isoforms were co-expressed along with PDCD5 in HEK-293T cells and interactions were assessed by co-immunoprecipitation and immunoblotting. PDCD5 associated with PhLP1 but not with Pdc, PhLP2A or PhLP3, confirming that a specific interaction between PhLP1 and PDCD5 was occurring (Figure 3-1A).

To determine whether the co-immunoprecipitation of PDCD5 with PhLP1 resulted from a direct interaction or from indirect interactions through other proteins in a common complex, the binding of recombinant purified PhLP1 and PDCD5 was tested in vitro by co-immunoprecipitation. Surprisingly, no PDCD5 was found in the PhLP1 immunoprecipitate whether PhLP1 was in its CK2 phosphorylated form (69) or not (Figure 3-1B). These findings suggest that PhLP1 and PDCD5 do not interact directly, but instead associate indirectly as components of the same complex.

Knowing that PhLP1 binds CCT, we investigated whether PDCD5 and PhLP1 were part of the same CCT complex. We first looked at the ability of PDCD5 to bind CCT by co-immunoprecipitation. PDCD5 immunoprecipitates contained significantly more endogenous CCTε than PhLP1 (Figure 3-1C). In a reciprocal experiment, both PDCD5 and PhLP1 were found in a CCT immunoprecipitate (Figure 3-1C). These results suggest that PhLP1 and PDCD5 might be interacting through CCT. To investigate this possibility, we measured the effect of
siRNA-mediated depletion of CCTζ on the ability of PhLP1 to co-immunoprecipitate PDCD5.

We found that an 80% depletion of CCTζ caused a corresponding decrease in the association of PDCD5 with PhLP1 (Figure 3-1D). This finding is consistent with the idea that PhLP1 and PDCD5 interact indirectly through the CCT complex. To confirm this result, we performed a double co-immunoprecipitation experiment. PhLP1 with a C-terminal tag consisting of a TEV cleavage site and a myc epitope was over-expressed along with FLAG-tagged PDCD5 in HEK-293T cells. PhLP1 was immunoprecipitated and released from the antibody and beads with TEV protease. The resulting supernatant was then subject to a second immunoprecipitation using a FLAG antibody. CCT was found in both the first and the second immunoprecipitates,
demonstrating that the same CCT complexes that were associated with PhLP1 were also bound to PDCD5 (Figure 3-1E). Collectively, these data show that PDCD5 does not bind PhLP1 directly but that they interact indirectly through a ternary complex with CCT.

**Functional Analysis of the PDCD5-CCT interaction**

The formation of a ternary complex between PhLP1, PDCD5 and CCT suggests that PDCD5 and PhLP1 may be functionally linked. To begin to test this possibility, we measured the effects of over-expression and siRNA-mediated depletion of PhLP1 or PDCD5 on the binding of the other to CCT. Surprisingly, neither over-expression nor depletion of PDCD5 had any effect on the interaction of PhLP1 with CCT in co-immunoprecipitation experiments (Figure 3-2 A and B). Likewise, over-expression or depletion of PhLP1 had no effect on PDCD5 binding to CCT (Figure 3-2C and D). These results indicate that PhLP1 and PDCD5 interact with CCT independently of each other, raising questions about the functional significance of PDCD5 binding to CCT. It does not appear that PDCD5 requires CCT and PhLP1 for folding because PDCD5 does not have the structure of a typical CCT substrate. It is a small 125 amino acid protein with a simple three-helical bundle fold without multiple domains or a complex folding pattern that are common among CCT substrates (144). Furthermore, PhLP1 has profound effects on the binding of its known substrate, Gβ, to CCT (69,95), but this was not observed with PDCD5 (Figure 3-2). These observations suggest that PDCD5 is not a CCT substrate but interacts with CCT for another reason. We tested this idea further by measuring the binding of nascent PDCD5 to CCT in a pulse-chase experimental format. Normally, nascent proteins that are CCT substrates rapidly bind to CCT upon synthesis and are released more slowly after folding (6,69). In contrast, nascent subunits of the CCT complex or co-chaperones like PhLP1 accumulate as part of CCT complexes over time. In a pulse-chase experiment, the CCT complex
was immunoprecipitated with an antibody to CCTε at increasing chase times after an [35S] methionine radiolabeling pulse, and the binding of nascent interacting partners was tracked over time (Figure 3-3A). Newly synthesized PDCD5 accumulated with the CCT complex, as did nascent CCT subunits, such as CCTα and γ. In contrast, nascent tubulin, a known CCT substrate (145), was released over time. These results show that PDCD5 is not a CCT folding substrate, but an interacting partner that could act as a co-chaperone or another type of regulator of CCT function.

To explore a possible co-chaperone function of PDCD5, we performed an analysis of PDCD5 binding partners. PDCD5 was immunoprecipitated in an ATP-depletion buffer to trap CCT substrates on the complex and potential interactors were identified by mass spectrometry (Table 3-2). Each of the CCT subunits was found, as were several isoforms of β-tubulin. The interaction between PDCD5 and β-tubulin was confirmed by co-immunoprecipitation. β-tubulin was clearly identified in PDCD5

![Figure 3-2. PhLP1 and PDCD5 bind CCT independently of each other.](image)

PDCD5 was either over-expressed (A) or knocked down (B), along with PhLP1-myc over-expression in HEK 293T cells. Cell lysates were immunoprecipitated with anti-CCTε (A) or anti-myc (B) and blotted as indicated. PhLP1 was either over-expressed (C) or knocked down (D), along with PDCD5-FLAG over-expression in HEK 293T cells. Cell lysates were immunoprecipitated with anti-CCTε (C) or anti-FLAG (D) and blotted as indicated. Bars represent the average ± S.E.M. from at least three experiments. Cell lysates were blotted for PDCD5-FLAG, endogenous PDCD5, PhLP1-myc, or endogenous PhLP1 as indicated to verify the over-expression and knockdowns. Representative blots are shown below the graphs.
immunoprecipitates while there was no specific interaction with α-tubulin (Figure 3-3B). The fact that both PDCD5 and β-tubulin interact with CCT suggests that they may form a co-complex on CCT. To test this possibility, we measured the effect of siRNA-mediated CCT depletion on the PDCD5-β-tubulin interaction (Figure 3-3C). An 80% reduction in CCTζ resulted in a 50% reduction in β-tubulin binding to PDCD5, suggesting that the PDCD5/β-tubulin interaction occurs at least in part through a co-complex with CCT. This interaction points to a possible role of PDCD5 in β-tubulin folding. To test this possibility, we measured the effect of PDCD5 knockdown on β-tubulin folding as well as several other known CCT substrates. To perform this measurement, we developed a new approach to assess tubulin folding by determining the rate of association of nascent α and β-tubulin with co-factor A (for β-tubulin) and co-factor B (for α-tubulin) in a pulse-chase experimental format. These co-factors are the first to interact with their

<table>
<thead>
<tr>
<th>Table 3-2. PDCD5 interacts with β-tubulin</th>
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U2OS cells were transfected with FLAG-TEV-PDCD5 or empty vector. PDCD5 immunoprecipitates were analyzed for binding partners by tandem mass spectrometry. The table displays significant hits found in the proteomics screen. The values in the first two columns indicate the normalized spectral counts for each sample. Values in the third column indicate the significance of the peptide hits (Decibans calculated from Bayes factors).
respective tubulins after they are folded by CCT in the process of tubulin dimer formation (146). For comparison, we also measured the effects of PDCD5 knockdown on β-actin and Gβ folding using previously established methods (57,64). PDCD5 knockdown increased the rate of β-tubulin

![Figure 3-3. PDCD5 inhibits β-Tubulin folding.](image)

A) The rate of association or dissociation from CCT complexes was measured by pulse-chase immunoprecipitations of CCTε from HEK 293T cells transfected with PDCD5-FLAG. The rate of association of CCT α and γ subunits (black, \( t_{1/2} = 112 \pm 18 \) min) and PDCD5 (red, \( t_{1/2} = 44 \pm 2 \) min) were calculated along with the rate of dissociation for tubulin (blue, \( t_{1/2} = 39 \pm 1 \) min). B) Binding of β-tubulin to PDCD5 was measured by co-immunoprecipitation from HEK 293T cells transfected with FLAG-PDCD5 or empty vector. C) The effect of CCT knockdown on β-tubulin binding to PDCD5 was measured by co-immunoprecipitation from HEK 293T cells treated with CCTζ siRNA or a control siRNA and later transfected with FLAG-PDCD5. The ratio of the β-tubulin band to the PDCD5 band was calculated and normalized to the control. D) The folding of the indicated proteins by CCT was measured by pulse-chase co-immunoprecipitations from HEK 293T cells treated with PDCD5 siRNA or negative control as indicated (see Experimental Procedures). E-F) The effect of PDCD5 knockdown (E) or over-expression (F) on β-tubulin binding to CCT was measured by co-immunoprecipitation with CCTε and immunoblotting as indicated. The ratio of the β-tubulin band to the CCTε band was calculated and normalized to the control. In all experiments, bars represent the average ± S.E.M. from at least three experiments. Representative gels or blots are shown below each graph. PDCD5 knockdown averaged between 65 and 80% as measured by immunoblotting.
folding by more than 50%, while it had no effect on α-tubulin, β-actin or Gβ folding (Figure 3-3D). These findings are consistent with the binding of PDCD5 and β-tubulin to CCT and indicate that PDCD5 interacts with CCT to specifically down regulate β-tubulin folding.

To further investigate the mechanism by which PDCD5 inhibited β-tubulin folding, we measured the effects of PDCD5 knockdown and over-expression on the binding of β-tubulin to CCT. An 80% siRNA reduction of PDCD5 increased the binding of β-tubulin to CCT by 35% (Figure 3-3E), while PDCD5 over-expression decreased the binding of β-tubulin to CCT by 40% (Figure 3-3F). In contrast, the binding of α-tubulin to CCT was unaffected by these changes in PDCD5 expression. Together, these findings indicate that PDCD5 specifically inhibits β-tubulin folding by disrupting the interaction of β-tubulin with CCT. This result was unexpected in light of the data from Figure 3-3B and 3-3C, showing that PDCD5 and β-tubulin form a complex with CCT. However, these observations can be reconciled if PDCD5 only partially inhibits β-tubulin binding to CCT (see Discussion).

Structural analysis of the PDCD5-CCT interaction

To begin to understand how PDCD5 might disrupt the interaction of β-tubulin with CCT, we performed a structural analysis of the PDCD5-CCT complex. An excess of purified PDCD5 was combined with purified CCT in vitro in the absence of nucleotide. The resulting complex was subjected to native gel electrophoresis after which the high molecular weight band corresponding to the CCT complex was excised and resolved on a denaturing gel. The denaturing gel showed the bands corresponding to the eight CCT subunits and a band corresponding to PDCD5, indicating the formation of a stable PDCD5-CCT complex (Figure 3-4A). The existence of the PDCD5-CCT complex was confirmed by electron microscopy (EM). Negatively stained EM images showed the typical doughnut-shaped structure corresponding to
end-on views of CCT (Figure 3-4B). Most of the particles revealed a small, elongated mass protruding into the CCT cavity. This mass was clearly observed upon processing and averaging of 1354 of these particles (Figure 3-4B), but was not present in the average image of 1128 PDCD5-free CCT particles (Figure 3-4C), thus the mass is attributable to PDCD5. The PDCD5 protruded from only one of the eight CCT subunits. To identify this CCT subunit, we employed an immuno-microscopy approach. A monoclonal antibody against CCTδ labeled the CCT subunit very near the PDCD5 mass, suggesting that PDCD5 binds CCTδ or an adjacent subunit (Figure 3-4D).

To determine its three-dimensional structure, the PDCD5-CCT complex was frozen-hydrated and subjected to cryo-EM. A three-dimensional reconstruction carried out with 13,000 particles to 25 Å resolution (Figure 3-5) revealed the typical barrel-shape structure built by the two octameric CCT rings in an open, substrate-receptive conformation, as is the case with CCT in the absence of nucleotide (Figure 3-6). The reconstruction clearly shows a small mass, attributable to PDCD5, protruding from one of the CCT subunits. Although

**Figure 3-4. PDCD5 binds CCT near the δ subunit.** A) Gel analysis of the PDCD5-CCT complex. PDCD5 was mixed with CCT at a 10:1 molar ratio in the absence of nucleotide and the mixture was resolved on a native polyacrylamide gel. The CCT oligomer (~960 kDa), which runs with a mobility clearly distinct from that of PDCD5 (~14 kDa), was excised and run on a denaturing acrylamide gel revealing bands corresponding to the eight CCT subunits and a band corresponding to PDCD5. B-D) Average electron microscopy images obtained from negatively stained particles. (B) PDCD5-CCT, (C) apo-CCT, (D) anti-CCTδ-PDCD5. Images were averaged from 1356, 1128 and 1018 particles, respectively.
The PDCD5 mass points toward the interior of CCT, it does not enter into the folding cavity but rather extends above it in a position not observed with CCT substrates actin and tubulin. A docking analysis using the 5.5 Å crystal structure of CCT in its open conformation (7) and an NMR solution structure of a PDCD5 fragment containing all but the last 12 residues of the C-terminus (144) gave a good fit into the cryo-EM three-dimensional reconstruction (Figure 3-6B). The facts that the mass attributed to PDCD5 accommodates the native atomic structure of the protein and that this mass is positioned on top of the folding cavity reinforce the idea that PDCD5 is not a substrate of CCT but rather may have a regulatory function.

The docking of the PDCD5 atomic structure was best when the C-terminus was oriented toward the CCT subunit (Figure 3-6B). To determine if the C-terminus of PDCD5 was involved in the interaction, the effects of several C-terminal truncations on PDCD5 binding to CCT were measured (Figure 3-6C). Near normal binding was observed for truncations up to the last nine residues.
residues (PDCD5 Δ117-125). However, further truncation (PDCD5 Δ115-125) resulted in a complete loss of binding. Thus, it is clear that the PDCD5 C-terminus participates in contacts with CCT, supporting the docking orientation shown in Figure 3-6B.

Chemical cross-linking of PDCD5 to CCT

The immuno-EM showed that PDCD5 was associated with CCTδ or an adjacent subunit. We sought to identify precisely which CCT subunit is involved by employing amber codon suppression technology to incorporate the photo-crosslinking unnatural amino acid p-benzoyl-L-phenylalanine (BzF) into residues at the C-terminus of PDCD5 and specifically cross-link the CCT subunit in close proximity to these residues (143). We engineered amber codons (TAG) at positions 115, 117 or 119 of the PDCD5 cDNA and transfected these variants into HEK-293 cells along with the BzF tRNA and BzF tRNA synthetase. Cells were subsequently incubated with BzF, and cell extracts were cross-linked with ultraviolet (UV) light (Figure 3-7A). PDCD5 was immunoprecipitated and immunoblotted to

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**Figure 3-6. PDCD5 binds the apical domain of one CCT subunit.** A) Two orthogonal views of the three-dimensional reconstruction of the PDCD5-CCT complex carried out with 13,000 particles at 25 Å resolution. B) The same two views showing the docking of the crystal structure of the open form of CCT colored by subunit (2XSM) and the atomic structure of PDCD5 in green (2K6B). C) Binding of various C-terminal truncations of PDCD5 to CCT was measured by co-immunoprecipitation and immunoblotting.
detect potential PDCD5 cross-links. Unfortunately, incorporation of BzF into PDCD5 was unusually low, less than 10% as judged by the intensity of the full-length, BzF- incorporated PDCD5 band in immunoblots of cell lysates compared to the truncated PDCD5 band resulting from failed incorporation (Figure 3-7B). In contrast, ~75% BzF incorporation was common for Gβ using this same method (Figure 3-7B). Despite the low degree of BzF incorporation, UV-induced PDCD5 cross-links were still observed but not as expected (Figure 3-8A). Surprisingly, we could detect a strong UV-dependent PDCD5 cross-link at ~75 kDa in the control sample, transfected with wild-type PDCD5 that had no amber codon incorporation site. This cross-link was also seen in the variants with amber codons at residues 117 and 119 producing the Δ117-125 and Δ119-125 truncations that retained binding to CCT, but the cross-link was greatly reduced in the residue 115 variant producing the Δ115-125 truncation that had lost binding to CCT. This unexpected result indicates that UV irradiation induced a PDCD5 cross-link independent of incorporation of BzF into the amber codon site.

Two observations suggested that the observed PDCD5 cross-link was to a CCT subunit. First, the cross-linking efficiency corresponded closely to the ability of the wild-type or truncated
PDCD5 variants to bind CCT (compare Figure 3-6C and Figure 3-8A). Second, the ~75 kDa size of the cross-link was approximately equal to the sum of the ~60 kDa mass of a CCT subunit and the 16 kDa mass of FLAG-tagged PDCD5. To further explore this possibility and to determine which CCT subunit might be involved, the cross-linked PDCD5 immunoprecipitates were immunoblotted for all eight CCT subunits. The ~75 kDa cross-link in the WT PDCD5 sample was detected only in the CCTβ immunoblot (Figure 3-8B). Furthermore, the CCTβ cross-link was also found only in immunoprecipitates of the WT, Δ117-125 and Δ119-125 PDCD5 variants that bound CCT and not with the Δ115-125 variants that did not bind CCT (Figure 3-8C). These results clearly show that PDCD5 was specifically cross-linked to CCTβ. Given that UV-induced cross-links are short lived and occur over distances of ~3 Å (143), PDCD5 must be in close proximity to CCTβ. This finding is consistent with the immuno-EM images showing that PDCD5 bound near CCTδ because in the recently revised orientation of the subunits within the CCT complex, CCTβ is adjacent to CCTδ (147,148).

From these results, it is not obvious how the UV-induced cross-linking of PDCD5 to CCTβ occurs, but the cross-linking was dependent on BzF and was independent of the BzF tRNA or synthetase. In cells not transfected with the BzF tRNA or synthetase, a robust PDCD5-CCTβ cross-link was observed in the presence of BzF, but not in its absence (Figure 3-8D). Thus, the free BzF in the cell extract must be catalyzing the cross-linking reaction. An examination of the free radical chemistry of BzF suggests possible ways that free BzF could cross-link two proteins. UV-light initiates a BzF diradical intermediate that then removes a hydrogen from an adjacent protein, creating an alkyl radical on the protein and a ketyl radical on the BzF. Normally, the two radicals then recombine to yield a benzhydrol modification of the protein (149). However in this case, a sufficient amount of the alkyl radical formed on either
PDCD5 or CCTβ must react with its binding partner to form a detectible inter-protein cross-link. This unusual radical chemistry confirms a close binding interaction between PDCD5 and CCTβ.

Figure 3-8. PDCD5 interacts directly with CCTβ. A) Cross-linking of PDCD5 to CCT. HEK 293T cells were transfected with the indicated FLAG-PDCD5 constructs, BzF tRNA, and BzF AA synthetase and treated with 1 mM BzF 3 hrs. post transfection. Lysates were exposed ± UV prior to immunoprecipitation and blotting for FLAG-PDCD5. The asterisk indicates a PDCD5 cross-linked band. HC – heavy chain. B) Cross-linking of PDCD5 to the CCT subunits was measured by co-immunoprecipitation from HEK 293T cells treated as in panel A and exposed to UV prior to immunoprecipitation with a CCTε antibody and blotting for each of the CCT subunits. The asterisk indicates a CCTβ cross-linked band. The arrows mark the position of the CCT subunits. NS – non-specific band. C) PDCD5 cross-linking to CCTβ depends on an interaction with the PDCD5 C-terminus. HEK 293T cells were treated as in panel A and lysates were exposed ± UV prior to immunoprecipitation with a CCTε antibody and blotting for CCTβ. The asterisk indicates a CCTβ cross-linked band. D) BzF catalyzes PDCD5 cross-linking to CCT without incorporation into Amber codon sites. HEK 293T cells were transfected with FLAG-PDCD5 (WT) in the absence of BzF tRNA and BzF AA synthetase. Cells were treated ± 1 mM BzF 3 hrs. post transfection. Lysates were exposed ± UV prior to immunoprecipitation with CCTε and immunoblotting for CCTβ. The asterisk indicates a CCTβ cross-linked band.
Discussion

Our search for novel PhLP1 binding partners has led to the serendipitous finding that the pro-apoptotic protein PDCD5 interacts with CCT. Investigation of the physiological role of the PDCD5-CCT interaction suggests that PDCD5 acts as a regulator of CCT function. The significant increase in β-tubulin folding upon PDCD5 depletion points to an inhibitory role for PDCD5 in β-tubulin folding (Figure 3-3D). This inhibition was specific for β-tubulin and was not shared with other CCT substrates α-tubulin, β-actin and Gβ. Accordingly, PDCD5 interfered with the binding of β-tubulin to CCT but not α-tubulin, indicating that PDCD5 specifically blocked β-tubulin folding by disrupting its interaction with CCT (Figure 3-3E and F). This disruption could result from a number of factors, but steric overlap of the binding sites of PDCD5 and β-tubulin on CCTβ seems likely. The cryo-EM reconstruction and cross-linking identifies an interaction of PDCD5 with the CCTβ helical protrusion at the tip of the apical domain (Figs. 3-4–3-8), and the crystal structure of the β-tubulin-CCT complex shows contacts between β-tubulin and the helical protrusion of CCTβ (7). A second β-tubulin contact site was also observed deeper within the CCT folding cavity, contacting the sensor loop of the equatorial domain (7). Thus, PDCD5 could interfere with β-tubulin binding in the helical protrusion without affecting its interaction with the sensor loop. Such partial inhibition is consistent with the observed effects of PDCD5 on β-tubulin binding to CCT and on β-tubulin folding (Figure 3-3).

The ability of PDCD5 to discriminate between α and β-tubulin was unexpected. The two proteins are structurally comparable and both interact similarly with CCT (7,10), yet PDCD5 only binds the β-tubulin-CCT complex and only inhibits β-tubulin folding (Figure 3-3). It appears that when α-tubulin is bound to CCT, PDCD5 is excluded and has no effect on α-tubulin
folding. The source of this specificity is unknown, but could result from PDCD5 disrupting CCT contacts specific to β-tubulin, or from a direct interaction between PDCD5 and β-tubulin. The residual binding of β-tubulin to PDCD5 upon CCT knockdown suggests that direct interactions between PDCD5 and β-tubulin do exist (Figure 3-3C).

PDCD5 has been proposed to act as a pro-apoptotic factor with tumor suppressor capabilities (132). However, the molecular mechanisms underlying its apoptotic function are largely unknown. Interactions between PDCD5 and two pro-apoptotic proteins, the histone acetyl transferase Tip60 (133) as well as the transcription factor p53 (134) have been proposed to contribute to its apoptotic role. A key question arising from our studies is what contribution PDCD5 binding to CCT and inhibition of β-tubulin folding contributes to PDCD5-mediated apoptosis. Interestingly, the C-terminal truncations of PDCD5 that were found to disrupt its interaction with CCT (Figure 3-6C) have been reported previously to be less effective in inducing cellular apoptosis (144). This correlation suggests that CCT binding and inhibition of β-tubulin folding could contribute to the apoptotic function of PDCD5. Tubulin dimer formation is a complex process, involving multiple co-factors that bring α- and β-tubulin together after their release from CCT (146). The process is vital for cell function in providing the building blocks for the microtubules that make the mitotic spindle and other important cellular structures. If mitotic spindle formation is disrupted, cells cannot divide and will eventually die, thus the large number of anti-cancer drugs that disrupt microtubule dynamics (150). PDCD5-mediated inhibition of β-tubulin folding could disrupt tubulin dimer formation and microtubule assembly and thus contribute to apoptosis.

The image emerging from this work and other previous studies shows CCT decorated with co-chaperones and regulators that modulate its protein folding function. For example,
prefoldin delivers actin and tubulin to CCT for folding (17). PhLP1 allows release of Gβ from CCT to associate with Gγ and form the Gβγ dimer (64), and now PDCD5 binds CCT to slow β-tubulin folding and possibly disrupt microtubule formation. By employing co-chaperones in this manner, CCT is able to expand and fine-tune its already versatile protein folding capacity.
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