Modulation of Phototropic Responsiveness in *Arabidopsis* through Ubiquitination of Phototropin 1 by the CUL3-Ring E3 Ubiquitin Ligase CRL3NPH3

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INTRODUCTION

Plants use sunlight not only for photosynthesis but also as a temporal and spatial cue for regulation of growth and development (Chen et al., 2004). A variety of adaptive responses have evolved such that plants can use light directionality, quantity, and quality to optimize their success. One such response is phototropism, or the bending of plant organs toward (stems and leaves) or away from (roots) a directional blue light (BL) source (Holland et al., 2009). The fitness benefits conferred to a plant by the phototropic response include maximization of photosynthetic light capture in aerial organs and water acquisition via roots (Pedmale et al., 2010).

Several key components of the phototropic signal response system have been identified and at least partially characterized. While the BL-activated Ser/Thr protein kinases, phototropin (phot1 and phot2, for a discussion of the nomenclature of the phototropins see, Briggs et al., 2001), are arguably the most notable proteins identified to date, the NONPHOTOTROPIC HYPOCOTYL3 (NPH3) protein is clearly the most enigmatic (Holland et al., 2009). The phototropins function as phototropic photoreceptors, with phot1 acting as the primary receptor under low-light intensities and both phot1 and phot2 functioning as redundant receptors under moderate to high light intensities (Sakai et al., 2001). By contrast, NPH3 appears necessary for phototropism under all BL conditions (Inada et al., 2004), though its biochemical function has remained elusive (Pedmale and Liscum, 2007).

NPH3 and ROOT PHOTOTROPISM2 (RPT2) represent the founding members of a novel plant-specific family of proteins (Motchoulski and Liscum, 1999; Sakai et al., 2000), designated the NRL (for NPH3/RPT2-Like) family (Holland et al., 2009). Several regions of sequence and predicted structural conservation define members of the NRL family, with three domains being most notable: (1) an N-terminal BTB (broad complex, tramtrack, bric à brac) domain, (2) a centrally located NPH3 domain (Pfam, PF03000), and (3) a C-terminal coiled-coil domain (Pedmale et al., 2010). The coiled-coil region of NPH3 has been shown to function as part of a phot1-interacting domain (Motchoulski and Liscum, 1999), but neither the BTB nor the NPH3 domain have been ascribed a biochemical function for any NRL family member. However, the BTB domains of NPH3 and RPT2 can mediate heterodimerization of these two proteins in yeast (Inada et al., 2004).

In recent years, a common functional role for the wide assortment of BTB domain–containing proteins (hereafter referred to as BTB proteins) has begun to emerge, namely, that BTB proteins...
act as both a CULIN3 (CUL3) binding and substrate adapter protein in CUL3-based E3 ubiquitin ligases (Willems et al., 2004). CUL-based E3 complexes, also called CRLs for CULIN-RING-ligases, catalyze the final step in a sequential three-enzyme process that results in the ubiquitination of a target protein (Hershko and Ciechanover, 1998). Though first described in fungal (Geyer et al., 2003) and animal cells (Pintard et al., 2003; Xu et al., 2003), CRL3s have been observed in plant cells as well (Dieterle et al., 2005; Figueroa et al., 2005; Gingerich et al., 2005; Christians et al., 2009).

Whereas proteolysis is the most commonly recognized outcome of CRL-dependent protein ubiquitination (Hershko and Ciechanover, 1998), protein ubiquitination also regulates a number of proteasome-independent cellular processes, including DNA repair and transcription, membrane protein endocytosis, and subcellular protein trafficking (Miranda and Sorkin, 2007; Chen and Sun, 2009). A number of ubiquitination patterns are also observed from one substrate to the next: a single ubiquitin (Ub) moiety can be ligated to a single Lys residue within the substrate protein (monoubiquitination), single Ub molecules can be attached to multiple Lys residues (multinubiquitination), and/or polyUb chains can be added at one or more Lys residues (polyubiquitination) (Komander, 2009).

The most well-characterized ubiquitination events are those using Lys-48 and Lys-63–linked polyUb chains, with the former being the most common proteasomal-targeting signal (Komander, 2009). Though mono/multiquibution has also been associated with protein turnover (Boutet et al., 2007), it is typically coupled to nonproteasomal functions. For example, in animal cells, activated plasma membrane receptor-ligand complexes are often mono/multiquibinated, internalized via clathrin-dependent endocytosis, and shuttled to the lysosome for destruction (Tanaka et al., 2008).

In this study, we show that the plasma membrane–associated phototropic receptor phot1 is ubiquitinated in response to BL activation. Ubiquitination of phot1 is dependent upon both the phot1–interacting proteins NPH3 and CUL3, suggesting that this posttranslational modification occurs via action of a CRL3NPH3 complex. Under high-light conditions, phot1 is both mono/multiquibinated and polyquibinated, with the latter stimulating the 26S proteasome–dependent degradation of phot1 likely as a means of sensor desensitization. By contrast, under low intensities of BL, phot1 appears to be exclusively monoquibinated, likely targeting the receptor for internalization. This latter hypothesis is certainly intriguing in light of recent findings that phot1 undergoes BL-induced clathrin-associated endocytosis (Kaiserli et al., 2009).

**RESULTS**

**NPH3 Is a CUL3-Interacting Protein**

In silico comparisons of the predicted tertiary structure of the NPH3 BTB domain with that of a number of plant BTB proteins known to interact with CUL3 (Dieterle et al., 2005; Figueroa et al., 2005; Gingerich et al., 2005; Christians et al., 2009) suggest that structural differences should not preclude NPH3-CUL3 interaction. Though previous studies failed to detect interaction between CUL3 and members of the NRL family by yeast two-hybrid or in vitro assays (Dieterle et al., 2005; Figueroa et al., 2005; Gingerich et al., 2005), we found that an N-terminal NPH3 polypeptide containing the complete BTB domain could be coimmunopurified with *Arabidopsis thaliana* CULLIN3a (CUL3a) when coexpressed in insect cells (see Supplemental Figure 1A online). Figure 1A shows that NPH3 can also be coimmunoprecipitated with CUL3a when both are coexpressed in mammalian COS-1 cells, confirming their potential for in vivo interaction. We found that full-length NPH3 interacts more strongly with CUL3a in COS-1 cells than any truncated version of NPH3 retaining the BTB domain (Figure 1A). However, the finding that the Flag–Δ1 polypeptide (lacking the BTB domain altogether) cannot interact with CUL3a (Figure 1A, lane 6) is consistent with the requirement of the BTB domain for CUL3 interaction. Moreover, the observed interaction between CUL3a and the Flag–hBTB polypeptide, which retains all of the predicted CUL3 binding sites (Stogios et al., 2005), demonstrates that amino acids within the C-terminal half of NPH3's BTB domain are sufficient to promote at least weak NPH3-CUL3 interaction (Figure 1A, lane 5). Interestingly, we found that NPH3 could also interact with human CUL3 in COS-1 cells and that this interaction exhibited a similar apparent requirement for both N- and C-terminal portions of NPH3 as that of the NPH3-CUL3a interaction (see Supplemental Figure 1B online). Thus, it appears that either portions within the C-terminal two-thirds of NPH3 are important for the structural integrity and CUL3 binding capacity of the BTB domain or that extra-BTB portions of NPH3 also play a direct role in CUL3 interaction. However, the latter scenario seems unlikely based on what is known about other BTB-CUL3 interactions (Stogios et al., 2005). Unlike NPH3, full-length RPT2 interacted more weakly with CUL3a in COS-1 cells (Figure 1A, lane 3). These latter findings are consistent with the weaker conditional phenotypes of the rpl2 mutants as well as RPT2’s apparent functional dependency on NPH3 (Sakai et al., 2000; Inada et al., 2004).

The findings that NPH3 and CUL3 can interact in nonplant cells prompted us to employ *Nicotiana benthamiana* transient coexpression system to ask if this interaction also occurs in plant cells. We first determined that transiently overexpressed NPH3 (with an mCherry tag) is associated with the plasma membrane in *N. benthamiana* leaf epidermal cells (Figures 2A and 2E), as occurs with native and transiently expressed NPH3 in other plant species (Motchoulski and Liscum, 1999; Lariguet et al., 2006; Inoue et al., 2008). Transiently expressed phot1, tagged with either green fluorescent protein (GFP) or mCherry, is also associated with the plasma membrane in *N. benthamiana* leaf epidermal cells (Figures 2F and 2I), as occurs with native and transiently expressed NPH3 in other plant species (Motchoulski and Liscum, 1999; Lariguet et al., 2006; Inoue et al., 2008). Transiently expressed phot1, tagged with either green fluorescent protein (GFP) or mCherry, is also associated with the plasma membrane (Figures 2F and 2I), as expected (Sakamoto and Briggs, 2002). When coexpressed, NPH3 and phot1 colocalize to the plasma membrane (Figure 2G). Moreover, NPH3 and phot1 are coimmunoprecipitated from microsomal fractions prepared from these transfected leaves (Figure 1B). The proper subcellular localization and physical interaction of NPH3 and phot1 in transfected *N. benthamiana* leaves suggests that native functions of these two proteins, including possible interaction and function with CUL3, can be examined in this system.

When CUL3a was transiently expressed in *N. benthamiana* leaves, we found that it localized to the nucleus and the plasma membrane (Figures 2B and 2J; see Supplemental Figure 2 online). These patterns of localization are consistent with observations in
mammalian cells, where CUL3 has been found to localize to the
nucleus, cytoplasm, Golgi, and plasma membrane (Singer et al.,
1999; Meyer-Schaller et al., 2009). Non-nuclear CUL3a colocal-
izes with phot1 and NPH3 (Figures 2C and 2K), indicating the
potential for in planta interaction between CUL3 and NPH3. In fact,
NPH3-mCherry commounprecipitates with GFP-CUL3a in mi-
crosomal fractions (Figure 1B) prepared from the N. benthamiana
leaves coexpressing these proteins (Figures 2A to 2C), confirming
not only that CUL3 can localize to the plasma membrane but also
that CUL3 and NPH3 can interact in planta.

**Normal Phototropism Requires Functional CUL3**

If NPH3 represents a bona fide CUL3-interacting protein, one
might expect mutations in CUL3 to result in altered NPH3-
dependent responses, such as phototropism. Single null loss-of-
function mutations in either of the CUL3 genes of *Arabidopsis*,
CUL3A or CUL3B, result in relatively subtle phenotypes, but
embryonic lethality is observed when both genes are knocked
out, indicating that the encoded proteins are essential and
functionally redundant (Figueroa et al., 2005). Fortunately, a
non-lethal hypomorphic double mutant (designated cul3<sup>hyp</sup>),
carrying a knockout allele of cul3b and a partial loss-of-function
allele of cul3a, exists; this double mutant facilitates functional
studies of postembryonic processes where functional redu-
dancy occurs (Thomann et al., 2009). Figure 3A shows that wild-
type, cul3a, and cul3b seedlings exposed to low-intensity BL
(0.1 μmol m<sup>2</sup> s<sup>−1</sup>) exhibited little difference in their hypocotyl
phototropic responsiveness. However, cul3<sup>hyp</sup> double mutants
grown under the same conditions exhibited significantly reduced
phototropism (Figure 3A). The fact that cul/+<sup>hyp</sup> remains more
responsive than the nph3-6 null mutant (Figure 3A) should not
be surprising since the former retains some CUL3 function
(Thomann et al., 2009). However, it would appear that the altered
phototropic response of the cul3<sup>hyp</sup> seedlings under low-light
conditions does not result from a general defect in hypocotyl
growth or tropic responsiveness (e.g., auxin responsiveness;
Harper et al., 2000) since all genotypes exhibited similar robust
gravitropic curvatures (Figure 3B).

When the phototropic response was examined under high-
intensity BL (120 μmol m<sup>2</sup> s<sup>−1</sup>), we found that in addition to the
cul3<sup>hyp</sup> double mutant, the cul3b single mutant also exhibited a
significantly diminished response (Figure 3C). Intriguingly, the
cul3 mutant lines exhibit a temporal-dependent phenotype
where more pronounced phototropic deficiency is observed early
in the response (Figure 3C). This suggests that either there
is a stronger requirement for CUL3 function during initial stages
of the phototropic response, or there is a cumulative effect of
retained CUL3 function over time, such that even the reduced
levels of CUL3 present in the cul3<sup>hyp</sup> double mutant are suffi-
cient to establish a nearly normal level of responsiveness by 6 h
of exposure to high light.

**BL Stimulates Ubiquitination of phot1 in Planta**

When taken together with the knowledge that BTB proteins
function as substrate adapters for CRL3s (Willems et al., 2004),
the findings presented here that NPH3 and CUL3 can interact in
vivo (Figure 1) and that CUL3 is required for normal phototropism
(Figure 3) suggest that ubiquitination of a substrate protein by a
CRL3<sup>NPH3</sup> complex likely represents an important component of
phototropic signaling. One obvious protein to examine for in
planta ubiquitination is phot1, since it is known to interact with

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**Figure 1. NPH3 Interacts with CUL3 in Vivo.**

(A) NPH3 interacts with CUL3 in mammalian cells. Flag-tagged NPH3
and RPT2 polypeptides coexpressed with HA-CUL3a in COS-1 cells are
shown on top. Constructs expressed the following NPH3 proteins: Flag-
NPH3, full-length NPH3 protein; Flag-ΔI, NPH3 from Pro-248 to terminal
Ser-746; Flag-hBTB, NPH3 from Pro-100 to terminal Ser-746; Flag-ΔCC,
NPH3 from Met-1 to Trp-650. Immunoblots of total cellular proteins
probed with anti-HA and anti-Flag antibodies are shown in the middle
two blots as controls for expression of the various constructs. The
bottom blot in uses immunoblotting (IB) with anti-Flag antibodies to
detect Flag-tagged proteins interacting with anti-HA antibody immuno-
precipitated (IP) HA-CUL3a. Blue dots denote the presence of a CUL3-
interacting Flag-tagged protein. aa, amino acids.

(B) Full-length NPH3 interacts with CUL3 in *N. benthamiana* cells. Two days
after infiltration, microsomal proteins were isolated from plants exhibiting
robust fusion protein expression (Figure 2). GFP-CUL3a and phot1-GFP
were immunoprecipitated (IP) with anti-GFP antibodies and then NPH3-
mCherry detected by immunoblot (IB) analysis with anti-DsRed antibodies.
Because a large number of proteins are likely to be ubiquitinated in a cell at any given time (Figure 4A, first lane; also see Stone et al., 2003), we used a transgenic line expressing a functional and immunoprecipitatable phot1-GFP fusion protein (Lariguet et al., 2006) to assess phot1 ubiquitination status specifically. As shown in Figure 4A, immunoblot analysis with an anti-Ub antibody (U5379; Sigma-Aldrich) detects a protein with similar mobility as immunoprecipitable phot1-GFP. Interestingly, a stronger immunoreactive signal was observed in samples from seedlings irradiated with low intensity BL (0.1 μmol m⁻² s⁻¹) than in those kept in darkness (Figure 4A, compare lanes 3 and 4 to lane 2), suggesting that not only is phot1 apparently ubiquitinated in planta but that BL influences this posttranslational modification. Because phot1 functions in phototropism over a wide range of light intensities (Sakai et al., 2001), we next determined if phot1 ubiquitination observed under low intensity BL (Figure 4A) also occurs at higher BL intensities. Figure 4B shows that phot1 is ubiquitinated over the same range of BL intensities for which it is active in promoting phototropism (Sakai et al., 2001), with the response being strongest at the highest intensity (120 μmol m⁻² s⁻¹).

Figure 2. Transiently Expressed NPH3, phot1, and Arabidopsis CUL3a Colocalize to the Cell Periphery in N. benthamiana Leaf Epidermal Cells.

(A) to (D) Localization of NPH3-mCherry ([A] and [C]) and GFP-CUL3a ([B] and [C]) signals to the periphery in tobacco cells. Signal overlap in (C) is colored yellow. (D) shows a bright-field image of the same cells shown in (A) to (C). White arrow in (B) and (C) indicates the nucleus.

(E) to (H) Localization of NPH3-mCherry ([E] and [G]) and phot1-GFP ([F] and [G]) signals to the cell periphery. Signal overlap in (G) is colored yellow. (H) shows a bright-field image of the same cells shown in (E) to (G).

(I) to (L) Localization of phot1-mCherry ([I] and [K]) and GFP-CUL3a ([J] and [K]) signals to the cell periphery. Signal overlap in (K) is colored yellow. (L) shows a bright-field image of the same cells shown in (I) and (J).
High-Intensity BL Stimulates Both Mono/Multi- and Polyubiquitination of phot1, with PolyUb-phot1 Being Targeted for Proteasome-Dependent Degradation

When the temporal dependency of phot1 ubiquitination under high-intensity BL (120 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) was examined, we observed a peak immunoreactive signal between 10 and 30 min after the start of irradiation, which declined and was largely undetectable after 4 h or more of irradiation (Figure 5A). It is worth noting that in Figure 5A, the ubiquitinated phot1 (phot1-Ub) is detected by an antibody (P4D1) that recognizes both mono/multi- and polyubiquitinated proteins. By contrast, when a polyubiquitin-specific antibody (FK1) is used, no such signal is observed in phot1-GFP immunoprecipitates (Figure 5B, lanes 1 to 6), though a large number of polyubiquitinated proteins are recognized in total protein extracts used for immunoprecipitations (Figure 5B, lane 8). Similar results were obtained with anti-K48 antibodies that specifically recognize K48-linked polyUb chains (Figure 5C), suggesting that phot1 is mono/multiubiquitinated rather than polyubiquitinated under high light conditions.

Previous studies by Sakamoto and Briggs (2002) showed that the abundance of phot1 protein declines after prolonged exposure to moderate intensity BL (20 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)), suggesting that the observed decrease in phot1-Ub signal under high-intensity BL (Figure 5A) might reflect a downregulation in steady state levels of phot1 itself. Immunoblot analysis with anti-phot1 antibodies shows that both membrane-associated and total cellular phot1 protein abundance does indeed decline in response to irradiation of seedlings with high-intensity BL and with a similar time course as that observed for the decrease in phot1-Ub signal (cf. Figures 6A and 6B to Figure 5A). The observed changes in phot1 abundance appear specific since NPH3 levels remain relatively constant (Figures 6A and B, bottom panels). BL-induced reduction in phot1 protein abundance is blocked by pretreatment of plants with the 26S proteasome–specific inhibitor MG132 (Figure 6C), indicating that phot1 is degraded by the 26S proteasome and that ubiquitination likely targets phot1 for destruction under high BL conditions.

Though not unknown, it is rather atypical for mono/multiubiquitinated proteins to be targeted for proteasome-dependent turnover (Boutet et al., 2007). Instead, polyUb chains (most commonly Lys-48 linked) represent the predominant molecular tag targeting proteins for degradation by the 26S proteasome (Komander, 2009). As such, we wondered whether our inability to detect polyubiquitinated phot1 (Figures 5B and 5C) might simply have been precluded by the degradation of phot1 under high-intensity BL, rather than lack of polyubiquitination per se. When plates for 3 d in darkness and then the plates were rotated on-edge 90° to induce a gravitropic response, as described previously (Stowe-Evans et al., 1998). Col, the wild type. Data represent the mean ± SE of at least 70 seedlings for each genotype. Student’s t test revealed that no genotype exhibited a response significantly different from that of the wild type (P > 0.05).

(C) Hypocotyl phototropism in seedlings exposed to high-intensity unilateral BL (120 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) for 2 (white bars), 4 (gray bars), or 6 h (black bars). Data represent the mean ± SE of at least 53 seedlings for each genotype. Col, the wild type. Mean responses differing significantly (P < 0.01; Student’s t test) from that of the wild type are indicated by asterisks.
that phot1 is both mono/multi- and polyubiquitinated under high
FK1, and K48 anti-Ub antibodies (Figures 5D to 5F), indicating
phot1-Ub is detected over the entire time course by the P4D1,
MG132 prior to exposure to high intensity BL (Figures 6A and 6B),
phot1 protein levels are stabilized by treating seedlings with
irradiated (0 h BL treatment) or exposed to low-intensity unilateral BL (0.1
Arabidopsis phot1-5PHOT1-GFP
from
Phot1 is ubiquitinated in planta. Total cellular proteins were prepared
immunoprecipitation are included as a positive control for total cellular
ubiquitination (Input, lane 1). Ubiquitinated proteins were detected in the
top panel by immunoblot (IB) analysis with U5379 anti-Ub antibodies
detected by immunoblot (IB) analysis with P4D1 anti-Ub antibodies.
with anti-GFP antibodies (lanes 1 to 7). Ubiquitinated proteins were
immunoprecipitation (IP) with anti-GFP antibodies (lanes 2 to 5). Total proteins not subjected to
immunoprecipitation are included as a positive control for total cellular
ubiquitination (Input, lane 1). Ubiquitinated proteins were detected in the
top panel by immunoblot (IB) analysis with US379 anti-Ub antibodies
(Sigma-Aldrich). Asterisk denotes the IgG heavy chain (50 kD) of the
GFP protein was detected in the bottom panel with anti-phot1 antibodies.
BL as a control. With the exception of an aliquot from the
phot1-5
sample;
that was retained as a positive control for IP samples. Phot1-
GFP protein was detected in the bottom panel with anti-phot1 antibodies.
BL stimulates ubiquitination of phot1 over a wide range of intensities.
Total cellular proteins were prepared from Arabidopsis phot1-5PHOT1-
GFP seedlings that had been mock irradiated (0 min) or exposed to 2 h of
unilateral BL at the indicated fluence rates, as well as from phot1-5
seedlings exposed to 2 h of BL at a moderate intensity (1.0 
BL as a control. With the exception of an aliquot from the
phot1-5 sample
that was retained as a positive control for total cellular ubiquitination
(Input, lane 8), all samples were subjected to immunoprecipitation (IP)
with anti-GFP antibodies (lanes 1 to 7). Ubiquitinated proteins were
detected by immunoblot (IB) analysis with P4D1 anti-Ub antibodies.
Asterisk denotes the IgG heavy chain (50 kD) of the
GFP antibodies and represents a loading control for IP samples.
phot1 protein levels are stabilized by treating seedlings with
MG132 prior to exposure to high intensity BL (Figures 6A and 6B),
phot1-Ub is detected over the entire time course by the P4D1,
FK1, and K48 anti-Ub antibodies (Figures 5D to 5F), indicating
that phot1 is both mono/multi- and polyubiquitinated under high

A Functional CRL3NPH3 Complex Is Required for Both Mono/
Multi- and Polyubiquitination, as Well as Subsequent
Degradation of phot1

Results presented thus far demonstrate that NPH3 and CUL3 are required, at least genetically, for normal phototropic
timeliness (Figure 3; also see Liscum and Briggs, 1995, 1996;
Motchoulski and Liscum, 1999), and phot1 is ubiquitinated in
response to BL (Figures 4 and 5). When taken together with the
knowledge that NPH3 also interacts with phot1 (Motchoulski and
Liscum, 1999; Lariguet et al., 2006; Ioue et al., 2008), these results
suggest that the regulatory functions of NPH3 and CUL3 on
phototropism may occur via a CRL3NPH3 complex-dependent
ubiquitination of phot1. In this context, it is compelling to note
that phot1 ubiquitination is not detected in the nph3-6-null mutant
under high BL conditions (Figure 7A). Moreover, high-BL-induced
phot1 protein degradation is dramatically retarded in both the
nph3 single and cul3null double mutant backgrounds (Figures 7B and 7C).

Low-Intensity BL Stimulates Only Mono/Multiubiquitination
of phot1

While it now seems clear that high intensity BL stimulates both
the mono/multi- and polyubiquitination of phot1 (Figure 5),
with the latter targeting the phot1 protein for 26S proteasomal
degradation (Figure 6), most studies on phot1-dependent pho-
tropism have been done under low-intensity BL conditions
(Motchoulski and Liscum, 1999; Harper et al., 2000; Sakai et al.,
2000, 2001; Blakeslee et al., 2004; Esmon et al., 2006; Lariguet
et al., 2006; Pedmale and Liscum, 2007; Kaiserli et al., 2009).
Therefore, we set out to examine the ubiquitination of phot1
occurring in response to low BL (Figure 4) in more detail. Though
the signal is not as intense as observed after irradiation with
high-intensity BL (Figure 5A), phot1-Ub is detected with the
P4D1 antibody in immunoprecipitated samples from PHOT1-
GFP seedlings exposed to as little as 10 min of low-intensity BL
(0.1 
BL as a control. With the exception of an aliquot from the
phot1-5 sample
that was retained as a positive control for IP samples. Phot1-
GFP protein was detected in the bottom panel with anti-phot1 antibodies.
BL stimulates ubiquitination of phot1 over a wide range of intensities.
Total cellular proteins were prepared from Arabidopsis phot1-5PHOT1-
GFP seedlings that had been mock irradiated (0 min) or exposed to 2 h of
unilateral BL at the indicated fluence rates, as well as from phot1-5
seedlings exposed to 2 h of BL at a moderate intensity (1.0 
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BL as a control. With the exception of an aliquot from the
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that was retained as a positive control for IP samples. Phot1-
GFP protein was detected in the bottom panel with anti-phot1 antibodies.
consequences of phot1 mono/multi- and polyubiquitination are likely to be different.

**DISCUSSION**

Eukaryotic cells frequently use ubiquitination as a posttranslational mechanism to regulate the stability, intracellular localization, and function of proteins in response to environmental signals (Gao and Karin, 2005; Miranda and Sorkin, 2007). Here, we show that BL stimulates the ubiquitination of the phototropic receptor phot1 (Figures 4, 5, and 8) and that this response is likely mediated by CRL3NPH3, a CUL3-based E3 ubiquitin ligase that uses the phot1-interacting BTB protein NPH3 as its substrate adapter (Figures 1, 2, 7, and 8E). Under high intensities of BL (e.g., 120 μmol m⁻² s⁻¹), phot1 appears both mono/multi- and polyubiquitinated (Figure 5), with the latter modification targeting...
From a biological perspective, it is worth noting that phot1 degradation has only been observed in seedlings exposed to intensities of BL equivalent to, or in excess of, that necessary for maximal phototropic response (Figure 6; see Supplemental Figure 3 online; Sakai et al., 2001; Sakamoto and Briggs, 2002; Pedmale and Liscum, 2007; Sullivan et al., 2010). This suggests that the stimulation of phot1 polyubiquitination under high BL conditions represents a means to desensitize phototropic signaling through receptor turnover. Based on this simple hypothesis, we predicted that mutants compromised in their CRL3<sup>NPH3</sup>-dependent phot1 degradation, such as <i>nph3-6</i> and <i>cul3<sup>hyp</sup></i> (Figures 7B and 7C), would exhibit enhanced phototropism in response to irradiation with high-intensity BL. However, significantly reduced phototropic

**Figure 6.** High-Intensity BL Stimulates 26S Proteasome–Dependent Degradation of phot1.

(A) Membrane-associated phot1 protein levels decrease in response to high-intensity BL. Microsomal proteins were prepared from wild-type (Col) Arabidopsis seedlings that had been mock irradiated (0 min) or exposed to high-intensity unilateral BL (120 μmol m<sup>-2</sup> s<sup>-1</sup>) for the indicated times. Top panel, phot1 was detected by immunoblot (IB) analysis with anti-phot1 antibodies; bottom panel, NPH3 was detected with anti-NPH3 antibodies as a stable loading control.

(B) Total cellular phot1 protein levels decrease in response to high-intensity BL. Wild-type (Col) seedlings were treated and phot1 and NPH3 detected as in (A).

(C) The proteasome inhibitor MG132 stabilizes phot1 protein levels in plants exposed to high-intensity BL. Microsomal proteins were prepared from wild-type (Col) seedlings treated with 50 μM MG132 for 2 h prior to being mock irradiated (0 min) or exposed to high-intensity unilateral BL (120 μmol m<sup>-2</sup> s<sup>-1</sup>) for the indicated times. Phot1 and NPH3 were detected by immunoblot (IB) analysis as described in (A).

**Figure 7.** CRL3<sup>NPH3</sup> Function Is Required for Ubiquitination and Degradation of phot1.

(A) High-intensity BL-dependent ubiquitination of phot1 is absent in the <i>nph3</i>-null mutant background. Total cellular proteins were prepared from <i>Arabidopsis</i> phot1-5<nph3>-6PHOT1-GFP seedlings that had been mock irradiated (0 min) or exposed to high-intensity unilateral BL (120 μmol m<sup>-2</sup> s<sup>-1</sup>) for the indicated times (lanes 1 to 6). Total cellular proteins were also prepared from mock irradiated <i>nph3-6</i> seedlings as a negative control (lane 7). In the top two panels, ubiquitinated proteins were detected by immunoblot (IB) analysis with P4D1 anti-Ub antibodies. Middle panel shows an extracted portion of the same blot from the top panel containing IgG heavy chain (~50 kD) from the α-GFP antibody (asterisk) as a loading control for IP samples. Bottom panel shows a replicate blot probed with anti-phot1 antibodies to demonstrate that although ubiquitinated phot1 is not detected by the P4D1 antibody (top panel), phot1-GFP is in fact immunoprecipitated effectively from phot1-5<nph3>-6PHOT1-GFP seedlings grown under high BL conditions.

(B) BL fails to stimulate phot1 degradation in the <i>nph3</i>-null mutant background. Microsomal proteins were prepared from <i>nph3-6</i> seedlings, and phot1 was detected in the top panel as described in (B). Bottom panel shows a replicate blot probed with an anti-syntaxin antibody (α-SYP) as a plasma membrane–specific protein loading control.

(C) BL-induced degradation of phot1 is retarded in the <i>cul3<sup>hyp</sup></i> mutant background. Microsomal proteins were prepared from <i>nph3-6</i> seedlings, and phot1 was detected by immunoblot (IB) analysis with anti-phot1 antibodies (top panel). Bottom panel shows a replicate blot probed with an anti-NPH3 antibody as a plasma membrane–specific protein loading control.
curvatures are observed in nph3-6 and cul3hyp mutants under high BL conditions (Figure 3C). While these findings do not in themselves argue against a connection between phot1 polyubiquitination and receptor desensitization, they do demonstrate that this simple model is not sufficient to explain the observed photophysiology. In this context, we would like to note that phototropism is severely impaired in nph3-6 and cul3hyp mutants in response to irradiation with low-intensity BL (Figure 3A), conditions which promote mono/multiubiquitination (Figures 8A and 8C), but not polyubiquitination (Figures 8B and 8D) or...
subsequent degradation of phot1 (Figure 8B; Sakamoto and Briggs, 2002; Pedmale and Liscum, 2007). These latter results suggest that mono/multiubiquination of phot1 positively influences functionality of the receptor relative to phototropism and that the abrogation of this effect in nph3-6 (Figure 8E) and cul3\(^{hyp}\) mutants masks any reduction in receptor desensitization that might occur due to the stabilization of phot1 protein levels in these mutants under high BL conditions (Figures 6B and 6C). Of course it also remains formally possible that polyubiquitination itself has a positive influence on phot1 quite apart from its proteasomal targeting activity. Additional studies are necessary to distinguish between these potential mechanisms.

Though we have just begun to characterize the low BL-stimulated mono/multiubiquitination of phot1, it seems clear, based on the mutant phenotypes just discussed, that this modification is important for normal receptor function, at least relative to phototropism. In fungal and animal systems, ligand-activated plasma membrane–associated receptors are often mono/multiubiquitinated to mark them for clathrin-dependent endocytosis as a means to modulate downstream signaling (d’Azzo et al., 2005; Miranda and Sorkin, 2007). Several pieces of independent data suggest that such a mechanism may provide a useful framework to help explain our results positively linking mono/multiubiquitination and phototropic function of phot1: First, BL induces the movement of phot1 from the plasma membrane to cytoplasmic locations (Sakamoto and Briggs, 2002). Moreover, pharmacological and immunoprecipitation studies suggest that BL-induced movement of phot1 is clathrin mediated (Kaiserli et al., 2009; Sullivan et al., 2010). We independently found that phot1 associates with clathrin heavy chain and that this association is enhanced by exposure to BL (see Supplemental Figure 4 online). Second, CRL3\(^{NPH3}\)-dependent mono/multiubiquitination of phot1 is stimulated by BL (Figures 4, 5, and 8), implying that photoexcited phot1 (i.e., receptor-ligand complex) is targeted more efficiently for ubiquitination than dark-state phot1 (i.e., free receptor). Lastly, it appears that phot1 activation by BL (i.e., formation of a receptor-ligand complex) is prerequisite to its clathrin-associated endocytosis (Kaiserli et al., 2009; Sullivan et al., 2010). We independently found that phot1 associates with clathrin heavy chain and that this association is enhanced by exposure to BL (see Supplemental Figure 4 online).}

**METHODS**

**Plant Materials and Growth Conditions**

Three-day-old dark-grown Arabidopsis thaliana seedlings used in this study were all in the Columbia (Col-0) accession. With the exception of the phot1-Snp3-6PHOT1-GFP line (gift from C. Fankhauser), all mutant and transgenic genotypes have been described previously; phot1-5 (Huala et al., 1997), phot1 phot2 (Kinosita et al., 2001), phot1-5PHOT1-GFP (Sakamoto and Briggs, 2002), nph3-6 (Motchoulakis and Liscum, 1999), cul3a-3 (Thomann et al., 2005), cul3b-1 (Thomann et al., 2009), and cul3\(^{hyp}\) (Thomann et al., 2009). The PHOT1-GFP transgene is functional and complements the phot1-5-null mutant phenotypes (Sakamoto and Briggs, 2002).

Arabidopsis seedlings were grown either on agar-based medium or in liquid culture (depending upon the experiment) as described below. First, seeds were surface sterilized using 30% (v/v) commercial bleach (20 min) and then washed five times with sterile water. Unless noted otherwise, seeds were next placed either on filter paper mounted on 1% (w/v) agar-solidified half-strength Murashige and Skoog (0.5 × MS) medium (Murashige and Skoog, 1962) in Petri plates or in 15 mL of 0.5 × MS liquid medium in 125- to 250-mL Erlenmeyer flasks. The seeds were then cold treated (4°C) for 3 d in darkness before treatment with red light for 1 h to induce uniform germination (Stowe-Evans et al., 1998). The plates or flasks were next transferred to complete darkness at 22 ± 2°C, and
seeding seeds were allowed to grow for 3 d prior to any treatment. For growth of seedlings in liquid medium, the flasks were shaken at 50 to 60 rpm throughout their growth and subsequent treatments up to tissue collection. For BL treatments, seedlings growing on agar medium were either mock irradiated or exposed to unilateral BL (Stowe-Evans et al., 1998) of various intensities for different time intervals, whereas seedlings grown in liquid medium were irradiated with BL at specified fluence rate from underneath the flasks with constant shaking (Pedmale and Liscum, 2007). Low-intensity BL (0.1 μmol m⁻² s⁻¹) was generated as described previously (Stowe-Evans et al., 1998), whereas moderate to high-intensity BL (10 to 120 μmol m⁻² s⁻¹) was generated by filtering light from a Navitar Xenon 560 projector (550-W xenon lamp) through one layer of blue acrylic (Rohm and Haas No. 2424, 3.18-mm thick; Cope Plastics). Intensities (fluence rates) were varied by altering the distance between the light source and plant material. For experiments employing proteasome inhibitor treatments (Lee and Goldberg, 1998), seedlings grown in liquid 0.5× MS medium supplemented with either the 50 μM MG132 (Sigma-Aldrich) or solvent in which the inhibitor was initially dissolved (DMSO) for 2 h prior (Feng et al., 2008) to irradiation or mock irradiation.

Four- to five-week-old greenhouse-grown Nicotiana benthamiana plants were used for transient expression of phot1, NPH3, and CUL3a and were grown from seed in the greenhouse under 16-h-light/8-h-dark conditions. Plants were watered twice weekly for the entire growth period. After infiltration plants were kept under continuous white light (~100 μmol m⁻² s⁻¹; Stowe-Evans et al., 1998) under laboratory conditions (22 to 24°C) until analyses were performed.

Gene Constructs

Unless otherwise noted, full-length PHOT1, NPH3, and CUL3a proteins (as described in TAIR; www.Arabidopsis.org) were expressed in insect and mammalian cells. In insect cells, we expressed only the first 188 amino acids of NPH3 (which includes the entire BTB domain; designated 6xHis-NPH3-BTB). In addition to full-length NPH3, various NPH3 truncations were expressed in mammalian cells (Figure 1A, top). Protein coding sequences were generated by PCR amplification from respective cDNA templates and cloned in frame with the indicated epitope tag for subsequent expression. Myc-phot1 was generated using the pCMV-Myc vector (Clontech); Flag-NPH3 (and all derivatives of NPH3; Figure 1A, top) and Flag-RPT2 with pCMV-tag2A (Stratagene); 6xHis-NPH3-BTB with pAcHLT-A (BD Biosciences); HA-At-CUL3a and HA-Hs-Cul3 with pCRUZ-HA (Santa Cruz Biotech) as described previously (Zheng et al., 2004); and GST-6xHis-At-CUL3a with pAcGHLT-A (BD Biosciences). The mouse Rbx1a construct used in insect cell studies has been described elsewhere (Li et al., 2005). Insect cell expression vectors used the Baculovirus polyhedrin promoter, while mammalian cell expression vectors used a CMV (cytomegalovirus) constitutive promoter.

For transient expression in N. benthamiana, GFP-CUL3a was generated by cloning the full-length coding region of Arabidopsis CUL3A (from cDNA) in frame with GFP using the pMDC43 vector (Curts and Grossniklaus, 2003). Similarly, phot1-GFP was constructed by cloning the full-length coding region of PHOT1 (from cDNA) in frame using the pMDC84 (Curts and Grossniklaus, 2003). A modified pMDC84 vector, designated pUP-Red, in which the GFP coding sequence was replaced with mCherry coding sequence (Shaner et al., 2004), was used to generate NPH3-mCherry and phot1-mCherry fusion proteins. The pUP vector construct was also engineered such that a Flag epitope tag is present on the fusion proteins, C-terminal to mCherry. All of the vectors for transient expression in N. benthamiana use a tandem duplicated cauliflower mosaic virus 3S5-derived constitutive promoter to drive fusion protein expression.

Insect and Mammalian Cell Culture and Transfections

The Trichoplusia ni cell line H5 (Invitrogen) was used for insect cell expression studies. Arabidopsis NPH3 (6xHis-NPH3-BTB) and CUL3a (GST-6xHis-CUL3a) polypeptides were coexpressed together with mouse Rbx1a in Hi5 cells according to established protocols (Li et al., 2005). The COS-1 African green monkey kidney cell line (ATCC) was used in all mammalian cell expression studies. COS-1 cells were grown in knockout Dulbecco’s modified Eagle’s medium supplemented with 15% ES-cell qualified bovine serum, 2.5 mM glutathione, and 100 μM mercaptoethanol. Transfections were performed with Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions.

Transient Expression of Proteins in N. benthamiana

For the transient expression of proteins in N. benthamiana, we used an established Agrobacterium tumefaciens-based infiltration method (Voinnet et al., 2003; Caplan et al., 2008). Each of the fusion protein expression vectors to be analyzed was first electroporated into the A. tumefaciens strain GV3101 (PMP90). Next, these A. tumefaciens cultures were grown overnight, pelleted, and resuspended in 10 mM MgCl₂, 10 mM MES and induced with 200 μM 4'-hydroxy-3',5'-dimethoxyacetophenone (acetrosyringone). An A. tumefaciens strain expressing the silencing suppressor protein P19 (Voinnet et al., 2003) was also prepared and coinfected with all of the test constructs. Leaves from 4- to 5-week-old N. benthamiana plants were infiltrated (Voinnet et al., 2003; Caplan et al., 2008) with a mixed suspension of A. tumefaciens (P19 strain plus one or more of strains harboring a test construct) in which each strain was adjusted to an optical density of 0.5 at 600 nm prior to mixing. Two days after infiltration, a small section of the infiltrated area was excised and used for confocal microscopy, or the entire leaf was used for protein extraction (see below).

Microscopy

Confocal fluorescence images were acquired on a Zeiss LSM 510 META NLO two-photon point-scanning confocal system mounted on an Axiovert 200M inverted microscope with a ×40/1.2 C-achromat water immersion objective. A 488-nm laser of the Zeiss LSM 510 META NLO two-photon point-scanning confocal system was used to excite GFP, and the fluorescent signal was visualized with a 500- to 550-nm band-pass filter. mCherry was excited with a 543-nm laser, and the emission signal was visualized with a 600- to 630-nm band-pass filter. Microscopy images were initially collected with the LSM510 operating software, and later the images were processed for brightness/contrast and overlaid with Adobe Photoshop CS3.

Preparation of Total Cellular and Microsomal Membrane Proteins from Arabidopsis and N. benthamiana

Total cellular and microsomal membrane proteins were isolated from Arabidopsis seedlings or N. benthamiana leaves after BL or inhibitor treatments essentially as described previously (Pedmale and Liscum, 2007). In brief, Arabidopsis seedling or tobacco leaf tissue was ground in ice-cold homogenization/resuspension buffer (50 mM HEPES, pH 7.8, 300 mM Suc, 150 mM NaCl₂, 10 mM NaAcetate, 5 mM EDTA, and 1× Plant Protease inhibitor cocktail [Sigma]), following by filtration through nylon cloth and centrifugation at 10,000g to remove particulate matter. Resultant total cellular proteins (supernatant of low speed centrifugation) were either retained for further use or subjected to ultracentrifugation at 100,000g for 1.25 h to pellet microsomes. Microsomal membranes were then resuspended in homogenization/resuspension buffer using a Potter-Elvenjem homogenizer. The resuspended microsomal membranes were aliquoted and stored in –70°C until used. Protein concentrations of samples were determined by the Bradford colorimetric method (Bio-Rad). All manipulations performed under dim red light in a 4°C temperature-controlled room.

Antibodies, Immunoprecipitation, and Immunoblot Analysis

The anti-NPH3 (Motchoulski and Liscum, 1999), anti-phot1 (Christie et al., 1998), and anti-SYP (Kalde et al., 2007) antibodies have been described elsewhere (Li et al., 2005). In Arabidopsis CUL3A, the NLO two-photon point-scanning confocal system was used to excite GFP, and the fluorescent signal was visualized with a 500- to 550-nm band-pass filter. mCherry was excited with a 543-nm laser, and the emission signal was visualized with a 600- to 630-nm band-pass filter. Microscopy images were initially collected with the LSM510 operating software, and later the images were processed for brightness/contrast and overlaid with Adobe Photoshop CS3.
previous experiments. Anti-6xHis (BD Biosciences), anti-Myc (Santa Cruz Biotechnology), anti-Flag (Sigma-Aldrich), Anti-DsRed/mCherry (Clontech), anti-GFP (Invitrogen), agarose-conjugated anti-GFP (MBL International), anti-HA (Convance), various anti-ubiquitin (US379, Sigma-Aldrich; 4D1, Cell Signaling Technology; FK1, Enzo Life Sciences; K48, Millipore; K63, eBio-science), as well as alkaline phosphatase (AP)-conjugated anti-rabbit IgG and anti-mouse IgG (Promega) antibodies were purchased from commercial sources. The 4D1 anti-Ub antibodies have been shown to detect both mono/multi- and polyubiquitinated proteins, whereas the FK1 anti-Ub antibodies detect only mono/multiubiquitinated proteins (Fujimuro et al., 1994; Haglund et al., 2003; Fujimuro and Yokosawa, 2005). Recently, Barberon et al. (2011) used the 4D1 and FK1 antibodies in studies of differentially ubiquitinated proteins in Arabidopsis, demonstrating the utility of these reagents in plants. In experiments using insect cells, glutathione affinity chromatography was employed to purify GST-6xHis-CUL3a and its interactions with Rbx1a and NPH3-BTB assessed through copurification and subsequent immunoblot analysis with the anti-6xHis antibody (Li et al., 2005). Prior to immunoprecipitation of expressed proteins from mammalian cells, total lysates from an aliquot of cells were collected (48 h after transfection) in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 0.1% bромphenol blue) and subjected to immunoblot analysis to assure relatively equal levels of expression of the various proteins of interest. For immunoprecipitation assays, mammalian cells were lysed in radioimmune precipitation assay buffer (10 mM sodium phosphate, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture [Sigma-Aldrich]). Cell lysates were preclared with Protein A beads and then incubated with 2 µg of appropriate affinity-purified antibody for 2 h at 4° C, and finally incubated for 2 h at 4° C with protein A-agarose beads. Immunoprecipitated proteins were washed three times with radioimmune precipitation assay buffer and then resuspended in SDS-PAGE sample buffer and boiled for 4 min before being subjected to immunoblot analysis. For immunoprecipitation of proteins from Arabidopsis and N. benthamiana, total and microsomal protein fractions prepared in homogenization/resuspension buffer with 1% Triton X-100 were handled as described previously (Lariguet et al., 2006). Protein samples were separated on SDS-polyacrylamide gels and then transferred to nitrocellulose membranes by electroblotting for subsequent immunodetection as described previously (Pedmale and Liscum, 2007). For immunodetection, membranes were first blocked using 5% (w/v) dry milk in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween 20 (Tris-buffered saline [TBS]/Tween) for 3 h. This was followed by incubation with appropriate primary antibody for 2 h in TBS/Tween plus 1% dry milk. Membranes were then washed three times with TBS/Tween, followed by incubation with appropriate AP-conjugated secondary antibody in TBS/Tween plus 1% dry milk for 3 h. Finally, membranes were washed four times with TBS/Tween (without dry milk), and primary antibody-reactive protein bands were detected by chemiluminescence using Immobilon AP substrate (Millipore).

Phototropism and Gravitropic Response Assays in Arabidopsis Seedlings

Arabidopsis seedlings were grown on horizontally (for phototropic assays) or vertically oriented (for gravitropic assays) 0.5 × MS agar-solidified media in darkness for 3 d and then assessed for phototropic or gravitropic responsiveness as described previously (Pedmale and Liscum, 2007).

Identification of Clathrin Heavy Chain as a phot1-Interacting Protein

Seedlings (phot1-5PHOT1-GFP) were grown on filter paper overlaid on 0.5 × MS medium for 3 d in darkness and then either mock irradiated or exposed to BL (10 µmol m⁻² s⁻¹) for 30 min. Microsomal membranes were then isolated according to methods previously described (Pedmale and Liscum, 2007). Microsomal pellets were resuspended in extraction buffer with 1% Triton X-100 and incubated on ice for 30 min. Samples were then centrifuged at 100,000g for 30 min at 4°C to pellet non-solubilized proteins. Resultant soluble microsomal membrane protein (250 µg per sample) was used for immunoprecipitation with the anti-GFP microbeads (Miltenyi Biotech) according to the manufacturer’s protocol. For the wash step, the manufacturer supplied lysis buffer was used, except that it was protease inhibitor treated before use. For elution, the native protein elution protocol was used, using 50 µL of 0.1 M triethylammonium, pH 11.8. Only one round of elution was performed. Samples were neutralized with 3 µL of 1 M MES, pH 3.0. Colloidal Coomassie Brilliant Blue−stained one-dimensional gel bands were excised and digested with trypsin, and samples were subjected to tandem time-of-flight analysis (Applied Biosystems 4700 Proteomics analyzer). A peptide mass fingerprinting search was conducted with the National Center for Biotechnology Information database to identify phot1-interacting proteins, one of which turned out to be clathrin heavy chain.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: PHOT1 (At3g45780), PHOT2 (At5g58140), NPH3 (At5g64330), RPT2 (At2g30520), Arabidopsis CUL3A (At1g26830), Arabidopsis CUL3B (At1g96670), human CUL3 (NM_003390), and clathrin heavy chain (At3g101130).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. NPH3-CUL3 Interactions in Insect and Animal Cells.

Supplemental Figure 2. Transiently Expressed GFP-CUL3A Appears to Associate with the Plasma Membrane in Plasmolyzed N. benthamiana Leaf Epidermal Cells.

Supplemental Figure 3. Phot1 Protein Is Stable under Low-Intensity Blue Light.

Supplemental Figure 4. Clathrin Heavy Chain Represents a phot1-Interacting Protein Whose Abundance Appears to Increase in Coimmunoprecipitates with phot1 in Response to in Planta Blue Light Exposure.

Supplemental References. References for Supplemental Figures 1 to 4.

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**AUTHOR CONTRIBUTIONS**

D.R., U.V.P., and E. Liscum designed the experiments, analyzed the data, and wrote the article. D.R. and U.V.P. performed the bulk of the experiments, and J.M. helped them. M.H. helped design human cell expression experiments, and U.V.P. performed them with S.S. X.T. and N.Z. performed the insect cell expression experiments. E. Lechner and P.G. provided seed stocks and critical phenotypic information prior to publication of cul3PHD mutant studies.

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**REFERENCES**


