Inositol hexakisphosphate kinase-1 mediates assembly/disassembly of the CRL4–signalosome complex to regulate DNA repair and cell death

Feng Rao^{a,1}, Jing Xu^{a,1}, A. Basit Khan^a, Moataz M. Gadalla^{a,b}, Jiyoun Y. Cha^a, Risheng Xu^{a,b}, Richa Tyagi^a, Yongjun Dang^{b,2}, Anutosh Chakraborty^{a,3}, and Solomon H. Snyder^{a,b,c,4}

^aThe Solomon H. Snyder Department of Neuroscience, ^bDepartment of Pharmacology and Molecular Sciences, and ^cDepartment of Psychiatry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Contributed by Solomon H. Snyder, September 19, 2014 (sent for review August 8, 2014)

Inositol polyphosphates containing an energetic pyrophosphate bond are formed primarily by a family of three inositol hexakisphosphate (IP6) kinases (IP6K1–3). The Cullin-RING ubiquitin ligases (CRLs) regulate diverse biological processes through substrate ubiquitylation. CRL4, comprising the scaffold Cullin 4A/B, the E2-interacting Roc1/2, and the adaptor protein damage-specific DNA-binding protein 1, is activated by DNA damage. Basal CRL4 activity is inhibited by binding to the COP9 signalosome (CSN). UV radiation and other stressors dissociate the complex, leading to E3 ligase activation, but signaling events that trigger signalosome dissociation from CRL4 have been unclear. In the present study, we show that, under basal conditions, IP6K1 forms a ternary complex with CSN and CRL4 in which IP6K1 and CRL4 are inactive. UV dissociates IP6K1 to generate IP7, which then dissociates CSN–CRL4 to activate CRL4. Thus, IP6K1 is a novel CRL4 subunit that transduces UV signals to mediate disassembly of the CRL4–CSN complex, thereby regulating nucleotide excision repair and cell death.

inositol phosphates | Cullin Ring E3 ligases | signalosome | UV radiation | DNA repair

Inositol pyrophosphates containing seven (IP7) or more phosphate groups on a myo-inositol ring are synthesized from inositol hexakisphosphate (IP6) primarily by a family of IP6 kinases that are conserved from yeast to humans and mediate diverse physiologic functions (1, 2). Among the three mammalian IP6K isoforms, IP6K1 and IP6K2 are widely distributed, whereas IP6K3 is expressed primarily in the brain (3). IP6K1 plays a role in diabetes (4), DNA homologous recombination (HR) repair (5), spermatogenesis (6), and chromatin modifications (7).

The Cullin–RING ubiquitin ligases (CRLs) control fundamental biological processes by mediating 20% of ubiquitin-dependent protein turnover (8). These E3 ligases are multiprotein complexes composed of the scaffold Cullins (Cul 1–7), the E2-interacting RING-finger protein Roc1/2, adaptor proteins specific for each Cullin family member, and adaptor-interacting substrate receptors that target substrates for ubiquitylation. CRL4, a DNA-damage–sensing member of this family (9), mediates the degradation of numerous substrates involved in cell cycle regulation (CDT1, p21, p27) as well as cell growth or death (c-Jun, p53) and is aberrantly active in many tumor types (10). The CRL4 complex comprises Cul4A/B and the adaptor protein damage-specific DNA binding protein 1 (DDB1). DDB1 binds to a family of WD40 domain-containing proteins that are substrate receptors (11, 12). The complex of DDB1 and its substrate receptor DDB2 binds directly to UV-damaged DNA to initiate nucleotide excision repair (NER) by ubiquitylating local histones and the NER machinery (9). Loss of DDB activity results in group E xeroderma pigmentosum, whose victims are hypersensitive to UV light. However, it remains unclear how CRL4 is activated by UV.

In plants, CRL4 is also regulated by UV. Thus, UV absorption by tryptophan residues in the dimer interface of UV-B Resistance 8 (UVR8) triggers dimer dissociation (13). Monomeric UVR8 binds and sequesters the CRL4 substrate receptor protein

Constitutively Photomorphogenic 1 (COP1), leading to stabilization of the CRL4 substrate elongated hypocotyl 5 (HY5), and HY5-like, members of the basic leucine-zipper (bZIP) family of transcription factors that are critical for UV-induced photomorphogenesis (14). A direct UV-sensing pathway has not been established in animals, presumably because animals lack photomorphogenesis as well as UVR8 homologs.

The CRLs are regulated by posttranslational modifications such as neddylation as well as protein–protein interactions with the signalosome or Cullin-Associated and Neddylation-Dissociated 1 (15). Neddylation of Cullins is required for their activity. The COP9 signalosome complex, comprising eight subunits (CSN 1–8), is highly conserved from plants to mammals (16) and inhibits CRL activity both as a deneddylase and as a direct Cullin-binding partner (17–19). However, genetic analysis reveals that CSN is required for proper CRL functioning in vivo (20), suggesting the importance of restraining basal CRL activity. Assembly and dissociation of the CSN–CRL4 complex is highly dynamic and substrate context-dependent (21, 22). UV radiation and other cell stressors elicit dissociation of this complex, leading to E3 ligase activation, which mediates diverse processes such as DNA replication arrest (23), chromatin remodeling (11), and NER (24). A major question, yet unanswered, is what triggers CSN–CRL4 dissociation (15, 17, 21).

Significance

Attachment of the small protein ubiquitin to other proteins, a process called “ubiquitylation,” triggers protein degradation. The Cullin and signalosome protein families cooperatively mediate this process, but how they are regulated has been obscure. We show that an inositol polyphosphate with seven phosphates, hence designated IP7, is critical. Under basal conditions, the enzyme that generates IP7 is bound to the Cullin/signalosome complex, which is thereby maintained in an inactive state. Stressful stimuli, such as UV radiation, stimulate the enzyme to form IP7, which dissociates the complex, leading to activation of the Cullins with attendant ubiquitylation and degradation of target proteins. This process may play a key role in how cells respond to environmental stressors.

Author contributions: F.R., J.X., and S.H.S. designed research; F.R., J.X., A.B.K., M.M.G., J.Y.C., and R.T. performed research; R.X., Y.D., and A.C. contributed new reagents/analytic tools; F.R., J.X., and M.M.G. analyzed data; and F.R. and S.H.S. wrote the paper.

The authors declare no conflict of interest.

¹F.R. and J.X. contributed equally to this work.

²Present address: Key Laboratory of Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, School of Basic Medical Science, Fudan University, Shanghai 200032, China.

³Present address: Department of Metabolism and Aging, The Scripps Research Institute, Jupiter, FL 33458.

⁴To whom correspondence should be addressed. Email: ssnyder@jhmi.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1417900111/-DCSupplemental.

Here we report that IP6K1 scaffolds the inert CSN–CRL4 complex through direct contact with DDB1 and CSN1/2, key components of the CRL4 and CSN complexes, respectively. Furthermore, IP6K1 functions as a UV-sensing module in this complex, triggering its disassembly via the generation of IP7.

Results

IP6K1 Interacts with CRL4 via Direct Binding to DDB1. Tandem affinity purification (TAP) followed by mass spectrometry reveals that IP6K1 interacts with DDB1 (Fig. 1*A* and Fig. S1*A*). Parallel experiments with tagged IP6K2 and IP6K3 fail to identify DDB1 (25). Both overexpressed (Fig. S1*B* and *C*) and endogenous IP6K1 coimmunoprecipitate with DDB1 (Fig. 1*B*). IP6K1, but not IP6K2, binds purified DDB1 in vitro (Fig. 1*C*). DDB1 is the CRL4 E3 ligase adaptor protein in complex with Cul4A/B and Roc1/2 (11). Pull-down of IP6K1 leads to coprecipitation of both overexpressed and endogenous Cul4A and Roc1 (Fig. 1*D* and Fig. S1*D*), indicating that IP6K1 interacts with the CRL4 complex. As we did not detect Cul4A/B or Roc1/2 by mass spectrometry in our TAP experiments, the binding to Cul4A and Roc1 is likely indirect and mediated by DDB1.

We wondered whether the catalytic activity of IP6K1 impacts its binding affinity for DDB1. Lysine 225 is critical for inositol phosphate binding by IP6K1, whereas serine 335 is located in the ATP-binding pocket (3). Both IP6K1-K225A and IP6K1-S335A bind to DDB1 more avidly than wild-type preparations (Fig. S1*E*). Binding is strongest between DDB1 and the IP6K1 double mutant, implying that catalytic activity of IP6K1 downregulates binding, an observation that is explicated below.

IP6K1 Inhibits CRL4 Substrate Ubiquitylation and Degradation. To probe the functional consequences of this IP6K1–CRL4 interaction, we explored whether CRL4 targets IP6K1 for proteasomal degradation by examining IP6K1 turnover via its rate of degradation in the presence of cycloheximide (Fig. S2*A–C*). IP6K1 appears to be stabilized by exposure to DDB1. Thus, the half-life of IP6K1 is prolonged with coexpression of Cul4/DDB1 (Fig. S2*A*), whereas depletion of DDB1 by siRNA leads to lower IP6K1 levels (Fig. S2*B*). Moreover, DDB1 knockdown by shRNA decreases levels of endogenous IP6K1 (Fig. S2*C*). Thus, IP6K1 is not degraded by CRL4 but instead is stabilized by binding to DDB1.

Fig. 1. IP6K1 directly binds to DDB1 and inhibits CRL4. (A) Tandem affinity purification revealed a 120-kDa protein as an IP6K1-interacting protein. Peptides identified by mass spectrometry are listed at the right of the box. (B) Confirmation of the interaction between IP6K1 and DDB1 by endogenous coimmunoprecipitation (ip) experiments. (C) Direct in vitro binding between purified HA–DDB1 and recombinant IP6K1, but not IP6K2. The immunoprecipitates were probed using a pan-IP6K antibody. (D) IP6K1 interacts with the DDB1–Cul4A–Roc1 E3 ligase complex. HEK293 cells were transfected with the plasmids as indicated. Cells were harvested 24 h after transfection and subjected to GST pull-down. Cul4A binds to IP6K1 as a doublet; the upper band was detected by anti-Nedd8 antibody.

Fig. 2. IP6K1 inhibits the ubiquitylation and degradation of CRL4 substrates. (A) Coexpression of IP6K1 prevents enhancement of ubiquitylation by the expression of DDB1/Cul4A. HEK293 cells in 60-mm plates were transfected with 1 μ g of IP6K1, DDB1, Cul4A, and ubiquitin or control vectors. Cell lysates were blotted after 24 h. (B) IP6K1 inhibits CRL4-mediated CDT1 ubiquitylation. HEK293 cells were transfected with the plasmids as indicated. At 24 h after transfection, cells were harvested and subjected to myc immunoprecipitation. (C) Levels of endogenous CDT1 in wild-type and IP6K1 knockout MEFs with/without UV treatment. (D–E) Levels of CRL4 substrates are diminished in IP6K1-depleted MEF (D) or HCT116 (E) cells. An asterisk (*) indicates a nonspecific band. (F) Increased c-Jun ubiquitylation in IP6K1-null MEFs. Amounts of proteins used for immunoprecipitation were adjusted based on c-Jun levels. (G) Western blot analysis of c-Jun levels after treatment with the indicated concentrations of MLN4924 (20 h).

We then examined whether IP6K1 regulates CRL4 E3 ligase. Stimulation of overall ubiquitylation by Cul4/DDB1 overexpression is abolished by coexpression of IP6K1 (Fig. 2*A*), indicating that IP6K1 inhibits CRL4. To characterize this inhibition, we first studied CDT1, a classical CRL4 substrate required for the initiation of DNA replication (23, 26). CDT1 ubiquitylation is markedly enhanced by overexpressing DDB1/Cul4A, an effect abolished in the presence of IP6K1 (Fig. 2*B*). UV triggers CRL4-mediated CDT1 degradation to arrest DNA replication and facilitate DNA repair (23, 26). This UV-induced CDT1 degradation is substantially more rapid in IP6K1-deleted murine embryonic fibroblasts (MEFs) (Fig. 2*C*), indicating enhanced CRL4 activity in the absence of IP6K1.

We also examined other substrates of CRL4, observing diminished levels of p27 and c-Jun (27, 28) in IP6K1 knockout MEFs (Fig. 2*D*). Levels of p21 and p53, two other substrates of CRL4 (29, 30), are also markedly reduced in IP6K1 knockdown HCT116 cells (Fig. 2*E*), suggesting that IP6K1 broadly inhibits basal CRL4 activity. For additional substantiation, we focused on the influence of IP6K1 upon the bZIP transcription factor c-Jun because its plant homologs, HY5 and HY5-like, are degraded by CRL4 in a UV-regulated manner (14). Diminished c-Jun levels in IP6K1-null MEFs do not stem from transcriptional deficits because basal levels of c-jun mRNA are similar in wild-type and IP6K1-null MEFs (Fig. S2*D*). Rather, ubiquitylation of c-Jun is markedly increased in IP6K1 knockout cells (Fig. 2*F*), suggesting that IP6K1 prevents c-Jun ubiquitylation and degradation. In

line with this notion, MLN-4924, a NEDD8-activating enzyme inhibitor that reduces the neddylation and catalytic activity of CRL4 (31, 32), dose-dependently increases levels of c-Jun in IP6K1-null, but not wild-type, MEFs (Fig. 2G). MLN4924 also rescues the diminished p53 and p21 levels in IP6K1 knockdown cells (Fig. S2E). These data suggest that IP6K1 physiologically inhibits substrate ubiquitylation and degradation by CRL4.

IP6K1 Mediates UV-Elicited Nucleotide Excision Repair and Apoptosis.

To elucidate physiological consequences of IP6K1 inhibition of CRL4, we examined the NER of cyclobutane pyrimidine (CPD) dimer, the major UV-induced photoproduct in DNA (9), as CRL4 plays a major role in NER (24). Both anti-CPD immunofluorescence staining and ELISA reveal significantly more rapid CPD dimer repair in IP6K1-deleted MEFs (Fig. 3A and B), which is again consistent with augmented CRL4 activity following IP6K1 deletion.

We then evaluated the influence of IP6K1 upon UV-induced apoptosis because CRL4 substrates such as p53 and c-Jun mediate apoptosis upon UV radiation (33–35). Compared with wild-type MEFs, IP6K1-null MEFs are markedly more resistant to UV-induced apoptosis, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, poly-ADP ribose polymerase (PARP) cleavage, Annexin-V staining, and TUNEL assay (Fig. 3C–F). These actions of IP6K1 are not restricted to fibroblasts. Thus, HEK293 cells with IP6K1 depleted by shRNA knockdown (Fig. 4B) display a 2.5-fold reduction in apoptosis (Fig. S3). The neddylation inhibitor MLN-4924 increases apoptosis of wild-type and IP6K1-deleted cells to the same extent (Fig. 3C), consistent with the notion that increased DDB1/Cul4A activity is responsible for diminished cell death in IP6K1-deleted preparations.

IP6K1 Inhibits CRL4 by Scaffolding the CSN–CRL4 Complex. Thus far we have shown that IP6K1 binds CRL4, maintaining it in an inactive state. We investigated mechanisms whereby IP6K1 regulates CRL4. IP6K1-bound Cul4A is enriched for a slow-migrating, modified form, which is detected by anti-Nedd8 antibody and thus appears to represent neddylated Cul4A (Fig. 1D). The signalosome, an eight-subunit (CSN 1–8) Cullin–deneddylase complex (16, 17), also preferentially binds neddylated Cullins (36)

(Fig. S4A). Accordingly, we explored the possibility that IP6K1 promotes complex formation between CRL4 and the signalosome (Fig. 4A). IP6K1 coimmunoprecipitates both CSN5 and CRL4, indicating the existence of an IP6K1–CRL4–CSN ternary complex. Moreover, IP6K1 expression enhances Cul4A's interaction with CSN2 and CSN5 (Fig. 4A), suggesting a role for IP6K1 in the formation of a CSN-bound, inactive CRL4. CRL4–CSN binding is stimulated more by kinase-dead than wild-type IP6K1. Catalytically inactive IP6K1 also binds more avidly than wild-type IP6K1 to DDB1/Cul4A and CSN, implying a more stable ternary complex. Conversely, IP6K1 knockdown (Fig. 4B and Fig. S4A) greatly diminishes CRL4–CSN binding, an effect rescued by expressing shRNA-resistant mouse IP6K1 in IP6K1 knockdown cells (Fig. S4B). The signalosome inhibits Cullins in part by enzymatic removal of the activating Nedd8 modification (17). Accordingly, we examined neddylation of Cul4A. IP6K1 depletion augments Cul4A neddylation (Fig. 4C). The binding of substrate receptor DDB2 to Cul4A is diminished upon IP6K1 depletion, suggesting that IP6K1 does not compete with the substrate receptor for DDB1 binding. Rather, as the signalosome can directly interact with substrate receptors (17), IP6K1 appears to promote the assembly of CRL4–CSN complexes incorporating substrate receptors.

How does IP6K1 enhance CRL4–CSN binding? One inositol polyphosphate kinase isoform, inositol 1,3,4-trisphosphate 5/6-kinase, directly associates with the signalosome (37). Apart from its interactions with DDB1, IP6K1 might also directly bind the signalosome. Consistent with this notion, IP6K1 retains substantial binding to CSN2 and CSN5 in DDB1-depleted cells (Fig. 4D), suggesting that IP6K1 can interact with the signalosome independently of DDB1. Furthermore, IP6K1–DDB1 interaction is markedly diminished in CSN2 knockdown cells (Fig. 4E), suggesting that lack of any one component of the ternary complex diminishes complex integrity.

To identify which subunit of the signalosome interacts with IP6K1, we examined IP6K1 binding to four subunits, chosen on the basis of their spatial proximity to the adaptor protein in a CSN–CRL1 electron microscopic structure (17). CSN2 and CSN1, which are structurally homologous to each other, coprecipitate with IP6K1 (Fig. 4F). The weak pull-down of CSN5 and CSN6 presumably reflects their integration into the signalosome

Fig. 3. IP6K1 mediates UV-elicited NER and apoptosis. (A) Immunofluorescence staining of CPD dimers in cells at indicated time points after UV treatment. (B) IP6K1^{-/-} MEFs are more efficient in nucleotide excision repair. Cells were harvested 24 h after UV irradiation and analyzed by ELISA for cyclobutane pyrimidine dimer. (C) Apoptosis of wild-type and IP6K1-null MEFs upon UV radiation, with or without MLN4924 pretreatment (0.5 μ M, 20 h) measured by MTT assay. (D) Apoptosis of MEFs measured by PARP cleavage. Lysates were blotted using both total PARP and cleaved PARP-specific antibodies. Arrow indicates cleaved PARP. (E) Apoptosis of MEFs measured by Annexin V-FITC flow cytometry analysis. (F) Apoptosis of MEFs measured by the TUNEL assay. Wild-type and IP6K1 knockout cells were irradiated with 200 J/m² UV and harvested 24 h after irradiation.

Fig. 4. IP6K1 is a scaffold that mediates association between the signalosome and CRL4. (A) IP6K1 overexpression promotes the association of CSN–Cul4A. The kinase-dead K225AK335A mutant of IP6K1 elicits formation of a more stable CSN–Cul4A complex. (B) shRNA knockdown of IP6K1 in HEK293 cells leads to reduced Cul4A–CSN binding. Asterisk indicates nonspecific band. (C) IP6K1 knockdown by siRNA augments neddylation of Cul4A. (D) IP6K1 binding to CSN2 and CSN5 in DDB1-depleted HEK293 cells. (E) IP6K1 binding to DDB1 in diminished in CSN2 knockdown cells. Asterisk indicates nonspecific band. (F) Coprecipitation of myc-IP6K1 and GST–CSN1/2/5/6. (G) Direct in vitro binding between HEK293-purified GST–CSN1/2 and *E. coli*-purified recombinant IP6K1.

holo-complex that binds IP6K1. Furthermore, recombinant CSN2 and, to some extent CSN1, purified from HEK293 cells (Fig. 4G) or *Escherichia coli* (Fig. S4 C and D), directly bind recombinant IP6K1 in vitro whereas CSN5 does not directly bind IP6K1 (Fig. S4C). Collectively, these data imply that IP6K1, independently of its catalytic activity, inhibits CRL4 by scaffolding the CSN–CRL4 complex wherein IP6K1 contacts both DDB1 and CSN1/2.

UV-Elicited CRL4–CSN Complex Disassembly Requires IP6K1. Having elucidated mechanisms whereby IP6K1 inhibits basal CRL activity, we wondered whether UV stimulates CRL4 by dissociating it from IP6K1. Consistent with prior literature, UV dissociates CSN from CRL4, leading to CRL4 neddylation (Fig. S5A). UV also dissociates IP6K1 from DDB1, Cul4, and CSN5, indicating that UV disassembles the ternary complex (Fig. 5 A and B). Notably, UV does not alter CSN–CRL4 binding in IP6K1 null cells (Fig. 5C), establishing that IP6K1 is an integral component of the CRL4–CSN complex that senses UV to trigger complex disassembly. In line with this notion, the augmented CSN–Cul4A interaction associated with IP6K1 coexpression is reversed upon UV treatment (Fig. S5B). IP6K1-null cells retain low levels of CRL4–CSN binding, apparently due to alternative modes of CRL4–CSN interaction.

As there are multiple points of contact in the IP6K1–CSN–CRL4 ternary complex, we sought to identify the binding interface(s) that responds directly to the UV stimulus. Although UV does not dissociate CRL4 from CSN in IP6K1 null cells (Fig. 5C), or IP6K1 from CSN in DDB1 knockdown cells (Fig. 5D), it readily dissociates IP6K1 from DDB1 in CSN2 knockdown cells (Fig. 5E). This finding implies that UV weakens interactions between IP6K1 and DDB1 to initiate complex dissociation.

IP7 Generation by UV-Activated IP6K1 Mediates Dissociation of the Cul4–CSN Complex. In trying to understand how IP6K1 regulates CRL4–CSN interactions, we were impressed by the greater efficacy of kinase-dead IP6K1 than wild-type IP6K1 in stabilizing the complex (Fig. 4A). This suggests that IP7 destabilizes the complex. Accordingly, we monitored binding of IP6K1 mutants to the signalosome as well as influences of UV radiation (Fig. 6A).

Radiation virtually abolishes binding of wild-type IP6K1 to the signalosome. This action is not evident with either of two catalytically inactive IP6K1 mutants, substantiating the importance of IP7 in regulating these interactions.

We then explored inhibition of IP6K1 with the potent and selective inhibitor *N*²-(*m*-(trifluoromethyl)benzyl)*N*⁶-(*p*-nitrobenzyl)

Fig. 5. IP6K1 mediates UV-induced dissociation of CRL4 and CSN. (A) UV radiation dissociates binding between endogenous IP6K1 and DDB1. Cells were lysed 20 min after irradiation. (B) UV radiation dissociates binding between IP6K1 and DDB1, Cul4A, or CSN5. Data from three separate experiments were quantified and presented as a bar graph (Right). (C) UV dissociates the Cul4A–CSN complex in wild-type but not IP6K1-null MEFs. (D) UV dissociates IP6K1–CSN5 binding in DDB1-proficient but not DDB1-deficient cells. (E) UV dissociates IP6K1–DDB1 binding in CSN2 knockdown cells.

purine (TNP) (25, 38) (Fig. S6A). TNP substantially increases Cul4A–CSN binding while decreasing Cul4 neddylation. TNP attenuates the UV-elicited dissociation of the Cul4/CSN complex (Fig. 6B), consistent with a role for IP7 in the dissociation event.

Enhancement of Cul4/CSN binding by IP6K1 inhibition raises the possibility that UV radiation stimulates the generation of IP7, which facilitates dissociation of the complex. We observe a doubling of IP7 formation with UV treatment of HEK293 cells (Fig. 6C) in which the great majority of IP7 is generated by IP6K1 (Fig. S6B). Given that UV dissociates IP6K1 from DDB1, we examined whether DDB1 inhibits IP6K1's catalytic activity. DDB1 does diminish IP6K1's conversion of IP6 to IP7 in a concentration-dependent manner in vitro (Fig. 6D and Fig. S6C). Together, these data indicate that UV releases IP6K1 from a DDB1-bound, inactive state, leading to generation of IP7 that promotes disassembly of the CSN–CRL4 complex.

Discussion

In the present study we demonstrate that IP6K1 binds and regulates the ubiquitin E3 ligase CRL4 in a complex with the signalosome and that IP6K1 is a signal transducer for stimulus-dependent CRL4 activation (Fig. 6E). The IP6Ks are evolutionarily conserved from primitive eukaryotes, such as *Entamoeba* (39), to humans, but are absent in plants (40). This distribution of IP6Ks complements that of UVR8, the plant UV sensor that regulates CRL4 (14). This suggests that IP6Ks, especially the IP6K1 homologs, are part of a conserved signaling axis that senses UV in animals.

The signalosome binds directly to Cullins, preventing their neddylation and binding to E2 or substrates and hence maintaining Cullins in an inactive state (17). One of the initiating steps in NER involves dissociation of the signalosome from CRL4, permitting its proteolytic DNA repair activities (21). Mechanisms determining the stability of the CRL4–CSN complex and its dissociation have been obscure. We show that, under basal conditions, IP6K1, acting in a noncatalytic fashion, maintains an inactive complex by direct binding to DDB1 and CSN1/2. In this complex, IP6K1 lacks catalytic activity. UV elicits IP6K1–DDB1 dissociation, which releases IP6K1 from DDB1 inhibition, thereby promoting IP7 generation. IP7 in turn dissociates the CRL4–CSN complex. This relay of dissociation events, coupled via the synthesis of IP7, might ensure that CRL4 is highly responsive to diverse stimuli, yet tightly regulated. IP7 appears to function like a canonical second messenger in this process, fulfilling criteria suggested by Shears et al. (41) for such a function. Molecular mechanisms whereby IP7 dissociates the signalosome–Cullin complex are unclear. Conceivably, the negatively charged phosphate of IP7 interacts with a conserved positively charged canyon surface of Cullins (42), eliciting conformational changes. IP7 fails to disrupt CRL4–CSN binding when added to cell lysates.

Fischer et al. (19) proposed a model for CRL4–CSN disassembly. They showed that, although addition of purified CSN inhibits the ubiquitylation of CRL4 substrate receptors in vitro, coaddition of CRL4 substrates reverses this inhibition, suggesting that substrate binding dislodges CSN, leading to CRL4 activation (19). Dependence of CRL4 activity on substrate encounter implies that CRL4 is essentially unregulated, which is inconsistent with in vivo data on rapid UV-dependent ubiquitylation and degradation of CDT1. Alternatively, CSN competing with substrates might represent one mechanism of restricting CRL4 activity, as suggested by Enchev et al. (17). Our finding that IP6K1 mediates the assembly of the CRL4–CSN complex affords a novel model for explaining CRL activation.

Contrary to the augmented NER that we observe in IP6K1 knockout cells, Bhandari et al. (6) reported diminished HR repair activity in IP6K1-deleted MEFs exposed to hydroxyurea, which creates double-strand DNA breaks. Repair mechanisms following double-stranded breaks (HR) differ from those following UV damage (NER). As the activity of CRL4 needs to be tightly controlled during HR repair (43, 44), uncontrolled CRL4 activity in IP6K1 knockout MEFs might impede HR repair

processes. Apart from mediating DNA-damage response, CRL4 targets numerous substrates important for spermatogenesis (45), histone remodeling (11), tuberous sclerosis (46), and circadian rhythms (47). Although IP6K1 is also implicated in some of these processes (6, 7), whether such actions involve CRL4 regulation has not been determined.

The Cullin family members interact with a variety of adaptor proteins. Except for DDB1, adaptors for all of the other members are BTB-fold proteins (15). Although the unique structure of DDB1 could underlie its regulation by IP6K1, IP6K1 may also influence other CRLs, given its association with the signalosome that regulates all CRLs. Indeed, one of the ubiquitylation substrates studied here, p27, is targeted by both CRL4 (27) and CRL1 (48). There are precedents for functional interactions between inositol phosphate signaling and the CRL/CSN system. In plants, receptors for auxin and jasmonate hormones are Cullin-based E3 ligases that contain structurally important IP6 and IP5 molecules, respectively (49). In mammals, inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome (37). Moreover, yeast IP6K (KCS1) displays negative genetic

Fig. 6. IP7 mediates the dissociation between the signalosome and CRL4. (A) UV-induced dissociation of CSN from wild-type IP6K1 but not the K225A or K225A/K335A mutants. (B) TNP pretreatment (5 μM, 0.5 h) prevents UV from abolishing Cul4–CSN binding. (C) UV increases levels of IP7 in HEK293 cells. Cells were labeled with [3 H]inositol for 3 d, irradiated with 100 J/m² UV, and cellular levels of inositol phosphates were analyzed after 30 min, as described in *Materials and Methods*. (D) DDB1 dose-dependently inhibits the catalytic activity of IP6K1. Reaction conditions were the following: 20 mM Tris (pH 7.5), 5 mM MgCl₂, IP6 (50 μM), 2 mM ATP, creatine phosphate (10 mM), creatine kinase (0.1 μg/μL), DTT (1 mM), IP6K1 (5 ng/μL), 32 °C, 30 min. Where purified DDB1 was used, its ratios to IP6K1 were 1:1, 3:1, or 9:1. Reactions were run using the PAGE method as previously described (25); samples were flanked by polyphosphate ladders. (E) Schematic depiction of the role of IP6K1 and IP7 in regulating the CSN–CRL4 complex. Under basal conditions, IP6K1 acts as a scaffold that promotes the formation of an inactive CSN–CRL4 complex wherein DDB1 inhibits IP6K1. In response to cell stressors such as UV, IP6K1 dissociates from DDB1, releasing it from inhibition by DDB1. The activated IP6K1 generates IP7, which further promotes the disassembly of the CRL4–CSN complex. CSN-free CRL4 is neddylated and functions as an active E3 ubiquitin ligase.

interactions with Cul4 (Rtt101) (50). Thus, inositol phosphates are evolutionarily linked to CRL E3 ligases.

Materials and Methods

MEF, HEK293, and 293T cells were cultured in standard condition (25). RNAi oligos were purchased from Qiagen. MLN04924 was obtained from Active Biochem. All other reagents were purchased from Sigma unless otherwise specified.

Plasmids. Plasmids pcDNA3-myc3-CUL4A, pcDNA3-FLAG-DDB1, pcDNA3-HA2-DDB1, pMD2.G, and pAX2 were from Addgene. IP6K1/2, DDB1, CSN1, CSN2, CSN5, and CSN6 were cloned into pGEX6p2 and pCMV-GST vectors at SalI/NotI restriction sites. Point mutants were made using a site-directed mutagenesis kit.

Tandem Affinity Purification. HEK293 cells overexpressing TAP-IP6K1 were lysed and processed as described for TAP-IP6K2 (25).

GST Pull-Down, Coimmunoprecipitation, and Western Blot. Cells lysates were prepared and immunoprecipitated, and Western blotting was performed and quantified as described before (25).

Expression and Purification of Recombinant Protein from *E. coli*. For GST-tagged protein (IP6K1 and DDB1) constructs, the plasmids were transformed into BL21 (DE3). Proteins were expressed and purified as described (25).

Inositol Profiling and IP6K Activity Assay. Radiolabeling with [3 H]inositol and inositol phosphate detection were done as previously described (4). The kinase activities of IP6K1 and its mutants were assayed in a 200- μ L reaction with the following: 20 mM Tris (pH 7.5), 5 mM MgCl₂, IP6 (50 μ M), 2 mM ATP, creatine phosphate (10 mM), creatine kinase (0.1 μ g/ μ L), DTT (1 mM), and IP6K1 (5 ng/ μ L).

Statistical Analysis. All results are presented as the mean and SE of at least three independent experiments. Statistical significance was calculated by Student *t* test (**P* < 0.05, ***P* < 0.01). Western blots are representative of three or more experimental replicates.

Immunocytochemistry, flow cytometry, ELISAs, and sources of antibodies are described in detail in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Drs P. Talalay and S. Wehage for sharing the UV irradiator. This work was supported by US Public Health Service Grant DA-000266 (to S.H.S.). M.M.G. is supported by National Institutes of Health Medical Scientist Training Program Award T32 GM007309.

- Chakraborty A, Kim S, Snyder SH (2011) Inositol pyrophosphates as mammalian cell signals. *Sci Signal* 4(188):re1.
- Barker CJ, Berggren PO (2013) New horizons in cellular regulation by inositol polyphosphates: Insights from the pancreatic β -cell. *Pharmacol Rev* 65(2):641–669.
- Saiardi A, Nagata E, Luo HR, Snowman AM, Snyder SH (2001) Identification and characterization of a novel inositol hexakisphosphate kinase. *J Biol Chem* 276(42):39179–39185.
- Chakraborty A, et al. (2010) Inositol pyrophosphates inhibit Akt signaling, thereby regulating insulin sensitivity and weight gain. *Cell* 143(6):897–910.
- Jadav RS, Chanduri MV, Sengupta S, Bhandari R (2013) Inositol pyrophosphate synthesis by inositol hexakisphosphate kinase 1 is required for homologous recombination repair. *J Biol Chem* 288(5):3312–3321.
- Bhandari R, Juluri KR, Resnick AC, Snyder SH (2008) Gene deletion of inositol hexakisphosphate kinase 1 reveals inositol pyrophosphate regulation of insulin secretion, growth, and spermiogenesis. *Proc Natl Acad Sci USA* 105(7):2349–2353.
- Burton A, Azevedo C, Andreassi C, Riccio A, Saiardi A (2013) Inositol pyrophosphates regulate JMJD2C-dependent histone demethylation. *Proc Natl Acad Sci USA* 110(47):18970–18975.
- Petroski MD, Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* 6(1):9–20.
- Scrima A, et al. (2011) Detecting UV-lesions in the genome: The modular CRL4 ubiquitin ligase does it best! *FEBS Lett* 585(18):2818–2825.
- Lee J, Zhou P (2012) Pathogenic role of the CRL4 ubiquitin ligase in human disease. *Front Oncol* 2:21.
- Higa LA, et al. (2006) CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. *Nat Cell Biol* 8(11):1277–1283.
- Jin J, Arias EE, Chen J, Harper JW, Walter JC (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol Cell* 23(5):709–721.
- Wu D, et al. (2012) Structural basis of ultraviolet-B perception by UVR8. *Nature* 484(7393):214–219.
- Huang X, et al. (2013) Conversion from CUL4-based COP1-SPA E3 apparatus to UVR8-COP1-SPA complexes underlies a distinct biochemical function of COP1 under UV-B. *Proc Natl Acad Sci USA* 110(41):16669–16674.
- Lydeard JR, Schulman BA, Harper JW (2013) Building and remodelling Cullin-RING E3 ubiquitin ligases. *EMBO Rep* 14(12):1050–1061.
- Wei N, Serino G, Deng XW (2008) The COP9 signalosome: More than a protease. *Trends Biochem Sci* 33(12):592–600.
- Enchev RI, et al. (2012) Structural basis for a reciprocal regulation between SCF and CSN. *Cell Reports* 2(3):616–627.
- Emberley ED, Mosadeghi R, Deshaies RJ (2012) Deconjugation of Nedd8 from Cul1 is directly regulated by Skp1-F-box and substrate, and the COP9 signalosome inhibits deneddylation of SCF by a noncatalytic mechanism. *J Biol Chem* 287(35):29679–29689.
- Fischer ES, et al. (2011) The molecular basis of CRL4DDB2/CSA ubiquitin ligase architecture, targeting, and activation. *Cell* 147(5):1024–1039.
- Wolf DA, Zhou C, Wee S (2003) The COP9 signalosome: An assembly and maintenance platform for cullin ubiquitin ligases? *Nat Cell Biol* 5(12):1029–1033.
- Hannss R, Dubiel W (2011) COP9 signalosome function in the DDR. *FEBS Lett* 585(18):2845–2852.
- Groisman R, et al. (2003) The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* 113(3):357–367.
- Hu J, McCall CM, Ohta T, Xiong Y (2004) Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage. *Nat Cell Biol* 6(10):1003–1009.
- Li J, et al. (2006) DNA damage binding protein component DDB1 participates in nucleotide excision repair through DDB2 DNA-binding and cullin 4A ubiquitin ligase activity. *Cancer Res* 66(17):8590–8597.
- Rao F, et al. (2014) Inositol pyrophosphates mediate the DNA-PK/ATM-p53 cell death pathway by regulating CK2 phosphorylation of Tti1/Tel2. *Mol Cell* 54(1):119–132.
- Higa LA, Mihaylov IS, Banks DP, Zheng J, Zhang H (2003) Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. *Nat Cell Biol* 5(11):1008–1015.
- Higa LA, et al. (2006) Involvement of CUL4 ubiquitin E3 ligases in regulating CDK inhibitors Dacapo/p27Kip1 and cyclin E degradation. *Cell Cycle* 5(1):71–77.
- Wertz IE, et al. (2004) Human de-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science* 303(5662):1371–1374.
- Dornan D, et al. (2004) The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* 429(6987):86–92.
- Abbas T, et al. (2008) PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdt2 ubiquitin ligase complex. *Genes Dev* 22(18):2496–2506.
- Soucy TA, et al. (2009) An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* 458(7239):732–736.
- Pan VW, et al. (2013) Ubiquitin E3 ligase CRL4(CDT2/DCAF2) as a potential chemotherapeutic target for ovarian surface epithelial cancer. *J Biol Chem* 288(41):29680–29691.
- Shaulian E, et al. (2000) The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell* 103(6):897–907.
- Tournier C, et al. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 288(5467):870–874.
- Milosevic J, Hoffarth S, Huber C, Schuler M (2003) The DNA damage-induced decrease of Bcl-2 is secondary to the activation of apoptotic effector caspases. *Oncogene* 22(44):6852–6856.
- Choo YY, et al. (2011) Characterization of the role of COP9 signalosome in regulating cullin E3 ubiquitin ligase activity. *Mol Biol Cell* 22(24):4706–4715.
- Sun Y, Wilson MP, Majerus PW (2002) Inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome by binding to CSN1. *J Biol Chem* 277(48):45759–45764.
- Padmanabhan U, Dollins DE, Fridy PC, York JD, Downes CP (2009) Characterization of a selective inhibitor of inositol hexakisphosphate kinases: Use in defining biological roles and metabolic relationships of inositol pyrophosphates. *J Biol Chem* 284(16):10571–10582.
- Wang H, DeRose EF, London RE, Shears SB (2014) IP6K structure and the molecular determinants of catalytic specificity in an inositol phosphate kinase family. *Nat Commun* 5:4178.
- Fassetti F, Leone O, Palopoli L, Rombo SE, Saiardi A (2011) IP6K gene identification in plant genomes by tag searching. *BMC Proc* 5 Suppl 2:S1.
- Shears SB, Gokhale NA, Wang H, Zaremba A (2011) Diphosphoinositol polyphosphates: What are the mechanisms? *Adv Enzyme Regul* 51(1):13–25.
- Kleiger G, Saha A, Lewis S, Kuhlman B, Deshaies RJ (2009) Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates. *Cell* 139(5):957–968.
- Cong F, et al. (2002) Interaction between UV-damaged DNA binding activity proteins and the c-Abl tyrosine kinase. *J Biol Chem* 277(38):34870–34878.
- Chen X, et al. (2006) A kinase-independent function of c-Abl in promoting proteolytic destruction of damaged DNA binding proteins. *Mol Cell* 22(4):489–499.
- Yin Y, et al. (2011) The E3 ubiquitin ligase Cullin 4A regulates meiotic progression in mouse spermatogenesis. *Dev Biol* 356(1):51–62.
- Hu J, et al. (2008) WD40 protein FBW5 promotes ubiquitination of tumor suppressor TSC2 by DDB1-CUL4-ROC1 ligase. *Genes Dev* 22(7):866–871.
- Ozturk N, VanVickle-Chavez SJ, Akileswaran L, Van Gelder RN, Sancar A (2013) Ramshackle (Brwd3) promotes light-induced ubiquitylation of Drosophila cryptochrome by DDB1-CUL4-ROC1 E3 ligase complex. *Proc Natl Acad Sci USA* 110(13):4980–4985.
- Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H (1999) p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* 9(12):661–664.
- Shabek N, Zheng N (2014) Plant ubiquitin ligases as signaling hubs. *Nat Struct Mol Biol* 21(4):293–296.
- Fiedler D, et al. (2009) Functional organization of the *S. cerevisiae* phosphorylation network. *Cell* 136(5):952–963.