

Inositol hexakisphosphate (IP6) generated by IP5K mediates cullin-COP9 signalosome interactions and CRL function

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The family of cullin-RING E3 Ligases (CRLs) and the constitutive photomorphogenesis 9 (COP9) signalosome (CSN) form dynamic complexes that mediate ubiquitylation of 20% of the proteome, yet regulation of their assembly/disassembly remains poorly understood. Inositol polyphosphates are highly conserved signaling molecules implicated in diverse cellular processes. We now report that inositol hexakisphosphate (IP6) is a major physiologic determinant of the CRL-CSN interface, which includes a hitherto unidentified electrostatic interaction between the N-terminal acidic tail of CSN subunit 2 (CSN2) and a conserved basic canyon on cullins. IP6, with an EC₅₀ of 20 nM, acts as an intermolecular “glue,” increasing cullin-CSN2 binding affinity by 30-fold, thereby promoting assembly of the inactive CRL-CSN complexes. The IP6 synthase, Ins(1,3,4,5,6)P5 2-kinase (IPPK/IP5K) binds to cullins. Depleting IP5K increases the percentage of neddylated, active Cul1 and Cul4A, and decreases levels of the Cul1/4A substrates p27 and p21. Besides dysregulating CRL-mediated cell proliferation and UV-induced apoptosis, IP5K depletion potentiates by 28-fold the cytotoxic effect of the neddylation inhibitor MLN4924. Thus, IP5K and IP6 are evolutionarily conserved components of the CRL-CSN system and are potential targets for cancer therapy in conjunction with MLN4924.

inositol hexakisphosphate | cullin RING E3 ligases | COP9 signalosome | IP5K | CRL

Cullin-RING ligases (CRLs), comprising cullins (Cul 1–3, 4A/B, 5, 7, 9), RING finger Roc1/2, and cullin-specific adaptors, form a prominent family of multiprotein E3 ubiquitin ligases that together mediate 20% of proteasomal degradation (1, 2), and are emerging therapeutic targets (3). CRLs require neddylation, the attachment of an ubiquitin-like NEDD8 molecule, for optimal function (4, 5) and are tightly regulated by the constitutive photomorphogenesis 9 (COP9) signalosome (CSN), an eight-subunit deneddylase complex conserved from plants to humans (6, 7). CSN biochemically inhibits but genetically activates CRLs (8–14). Elucidating the mechanism of binding and dynamic disassembly of the CRL-CSN complexes is therefore key to understand physiological functions of CRLs (6). Despite recent progress in solving the crystal structures of CRLs (1, 15), CSN (16), and the electron microscopy structure of the CSN-CRL1 complex (10), structural elements mediating CRL-CSN interactions remain elusive. Moreover, the molecular switches underlying CRL-CSN complex dynamics are unclear.

The inositol polyphosphate pathway interacts with the CRL-CSN complexes via yet unspecified molecular mechanisms. Inositol polyphosphates (IP4, IP5, IP6) are highly conserved signaling molecules generated from the second messenger inositol 1,4,5-trisphosphate (IP3) by a family of inositol phosphate kinases (IPKs), including inositol 1,4,5-trisphosphate 3-kinases, inositol 1,3,4-trisphosphate 5/6-kinase (ITPK1), inositol polyphosphate

multikinase (IPMK), and inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IP5K) (17). Majerus and colleagues reported that CSN copurifies with ITPK1 (18), which catalyzes the first committed step in forming higher inositol polyphosphates. However, the physiological significance of the ITPK1-CSN interaction remains unclear (19). IP6 can be further phosphorylated by IP6 kinases (IP6Ks) and diphosphoinositol pentakisphosphate kinases (PIIP5Ks) to generate highly energetic inositol pyrophosphate species (IP7 and IP8) (20). Recently, we reported that IP6 kinase-1 (IP6K1) binds both CSN and CRL4A to promote CRL4A-CSN complex formation in a catalytic activity-independent manner (21). Both kinase-dead IP6K1 and the IP6K inhibitor TNP [N(2)-(m-(trifluoromethyl)benzyl)N(6)-(p-nitrobenzyl)purine] further stabilize the CRL4A-CSN complex, suggesting that the catalytic activity of IP6K1 (i.e., the turnover of IP6), negatively regulates CRL4A-CSN complex stability. However, the mechanism of action of IP6 and its related metabolites remains unknown.

In the present study, we delineate CRL-CSN interaction mechanisms, demonstrating an essential electrostatic interaction

Significance

The regulation of ubiquitylation is critical to maintain proteomic and cellular homeostasis. The cullin-RING E3 ubiquitin ligases (CRLs) mediate one-fifth of all ubiquitylation, but their regulation is largely unknown. Here, we describe how the generation of a small metabolite, inositol hexakisphosphate (IP6), locally switches two CRLs from their active to their inactive state by means of stabilizing their interaction with an inhibitor: the constitutive photomorphogenesis 9 signalosome. Furthermore, we demonstrate the physiologic consequences of IP6 depletion on CRL dysregulation, CRL substrate levels, and global cellular phenotypes. Targeting IP6 synthesis synergizes with the cytotoxic effect of CRL inhibition, which may have therapeutic relevance.

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Conflict of interest statement: Dr. York and the authors have independently supplied reagents to the same laboratory and have therefore appeared as coauthors in a paper published as Pulloor NK, et al. (2014) Human genome-wide RNAi screen identifies an essential role for inositol pyrophosphates in type-I interferon response. *PLoS Pathog* 10(2):e1003981, although we did so independently and without knowledge of the other's participation in the work.

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with CSN2. Cul4A K465E and K675E mutants no longer bind CSN2 or CSN5, whereas Cul4A N-terminal binding to adaptor DDB1 (24) is not affected (Fig. 1G). Purified Cul4A K465E and K675E proteins manifest diminished ability to directly pull down recombinant CSN2 in vitro (Fig. S2G). Mutating other residues (K661/3E) in the basic canyon also abolishes interactions with CSN (Fig. S2H). Taken together, these data establish the importance of the basic canyon in Cul4A for binding to CSN2.

IP6 Augments Binding of Cullins to CSN2. We hypothesized that highly charged inositol polyphosphates disrupt the electrostatic CRL–CSN2 interactions, thereby providing a regulatory means to control CRL–CSN complex disassembly. Unexpectedly, IP6 and IP7 markedly enhance Cul4A binding to the purified CSN holo-complex (Fig. 2A). IP6/7 acts directly on the cullin–CSN2 interface, because Cul4A–CSN2 binding is also augmented by IP6/7 (Fig. 2B), but not by inositol hexakis-sulfate (IS6) (Fig. S3A), or IP4 and IP5 isomers, except for some modest effects of Ins(1,2,3,5,6)P5 (IP5₄) (Fig. S3B). IP6/7 also augments in vitro binding of CSN2 to Cul1 and to some extent Cul2 (Fig. 2C and D), but not Cul3 (Fig. S3C).

IP6 dose–response analysis reveals significant effects at 5 nM IP6 and maximal actions at 200 nM (Fig. 2E and Fig. S3D), concentrations substantially lower than physiologic, intracellular IP6 levels, which range from 10 to 80 μ M (25). Other IP6-binding modules, where IP6 often acts as intermolecular bridge, have similarly high affinity (26–30). IP7 is somewhat less potent than IP6 (Fig. 2E), with EC_{50} values for IP7 (70 nM) about threefold higher than for IP6 (23 nM). Because intracellular IP7 concentration is more than 10-fold lower than IP6 (20), the weaker potency of IP7 suggests that IP6 is the physiologic mediator. To estimate the efficacy of IP6 at promoting CSN2–Cul4A binding, we fixed IP6 concentration at 1 μ M (saturating) and performed rCSN2 concentration–response analysis, which reveals a marked left-shift in the presence of IP6, with a 29-fold increase in affinity (Fig. 2F).

IP5K Binds to CRLs and Generates Physiologic IP6 to Mediate CRL–CSN Complex Stability. Thus far we have shown that IP6 potently and efficiently increases the affinity of certain cullin–CSN2 complexes in purified systems. To assess whether IP6 is physiologically required for CSN2–cullin binding, we examined Ins(1,3,4,5,6)P5 2-Kinase (IP5K/IPPK), the only enzyme that generates IP6 in human

cells (17). IP5K (Fig. 3A), but not the inositol pyrophosphate-generating PPIP5Ks (Fig. S4A), coprecipitates with CSN and cullins. Kinase-dead IP5K, IP5K-K138A (31) pulls down Cul4A and DDB1 equally well but only weakly pulls down CSN2/5 (Fig. 3B), suggesting that IP5K binds directly to CRLs but not CSN. Further fragment mapping experiments suggest that the N-terminal domain of Cul4A, which mediates binding to the adaptor protein DDB1 (24), is not required for binding IP5K (Fig. 3C). Indeed, using purified recombinant proteins, we observe direct interactions between the C-terminal domain of Cul4A (amino acids 401–end) and IP5K (Fig. 3D). Taken together, these data indicate that IP5K binds to CRL in a kinase-independent fashion, locally generating IP6, which augments CRL–CSN binding in a kinase-dependent fashion.

We previously reported that the IP6 metabolizing enzyme IP6K1 binds to CRL4A to promote the formation of the inactive CRL4A–CSN complex (21). Furthermore, this interaction is disrupted by UV radiation, leading to CRL activation (21). In contrast to IP6K1, IP5K binding to Cul4A is not altered upon UV stimulation (Fig. S4B), suggesting that IP5K constitutively associates with Cul4A, and that IP6 is basally required for CRL4A–CSN interaction.

To further validate the role of IP5K, we examined its depletion. shRNA targeting IP5K depletes IP5K mRNA (Fig. S4C), protein, and IP6 levels (Fig. 3E). This depletion of IP5K also markedly diminishes binding of CSN to both endogenous (Fig. 3F) and overexpressed (Fig. 3G) Cul4A, but not Cul3, without affecting interactions among CSN subunits (Fig. 3F). Binding of CSN2 to Cul1 and Cul2 is also decreased with IP5K depletion (Fig. S4D). Taken together, these data suggest that IP5K differentially influences specific cullin–CSN complexes in a manner consistent with the observed effects of IP6 on these interactions in vitro (Fig. 2B–D and Fig. S3C).

To confirm that IP5K knockdown acts by depleting IP6, we supplemented cell lysates with exogenous IP6. Adding IP6 to cell lysates with IP5K knockdown, but not control cell lysates, dose-dependently rescues Cul4A–CSN binding (Fig. 3H), with minimal effect on Cul3–CSN interactions (Fig. S4E). The effect of IP6 is unlikely to be caused by its downstream metabolites because it is stable under the assay conditions (Fig. S4F). These data establish that IP6 physiologically mediates specific CSN–CRL interactions. Finally, treating affinity-precipitated GST–Cul4A with bacterial phytase, an IP6 phosphatase, dissociates CSN from CRL4A (Fig. 3I),

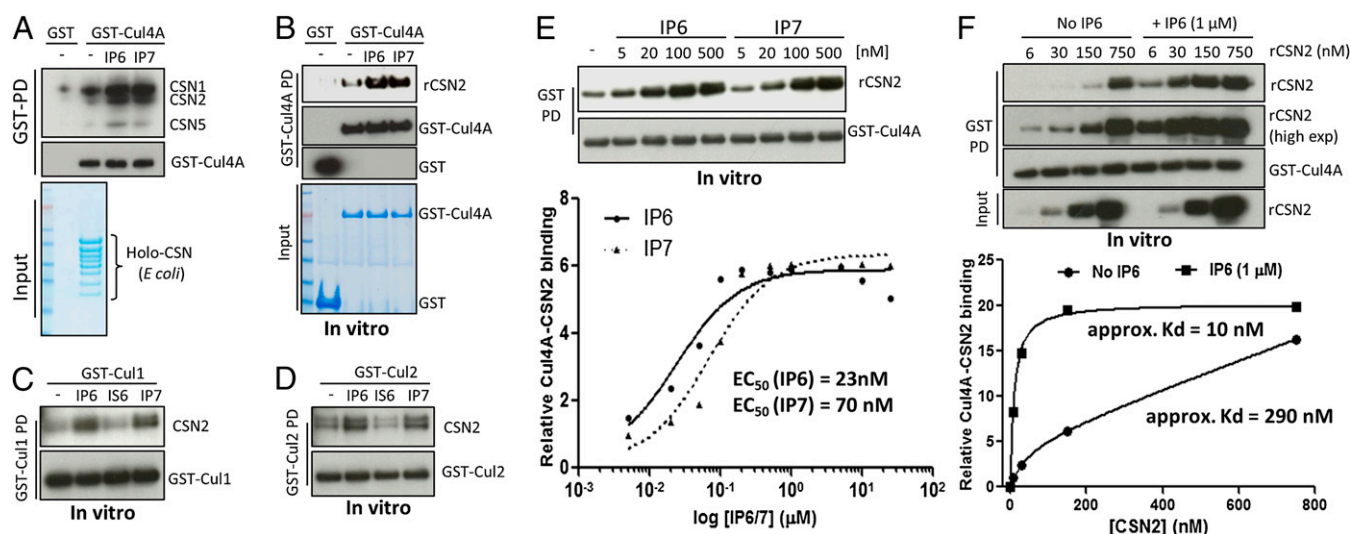


Fig. 2. IP6 stimulates CSN2–Cul4A binding in vitro. (A) IP6 and IP7 stimulate in vitro binding between HEK293-purified Cul4A and recombinant holo-signalosome. GST–Cul4A pull-down was blotted for CSN1, -2, and -5 simultaneously. (B) IP6 and IP7 stimulate in vitro binding between purified Cul4A and recombinant CSN2. (C and D) IP6 stimulates binding between recombinant CSN2 and HEK293-purified Cul1 (C) and Cul2 (D). (E) Concentration-dependent analysis of IP6 in stimulating CSN2–Cul4A binding. (F) CSN2 concentration-dependent analysis of CSN2–Cul4A binding with/without IP6.

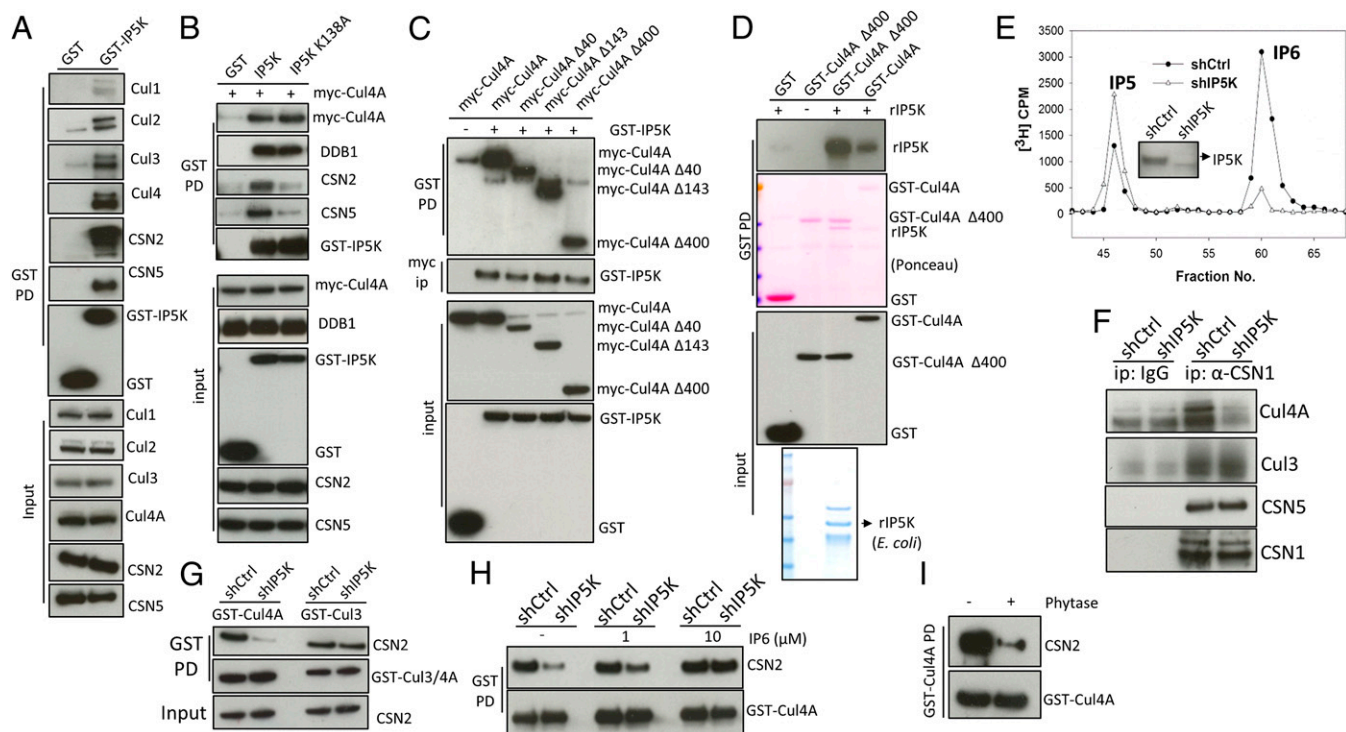


Fig. 3. IP5K binds CRL, generating IP6 to assemble CRL–CSN complexes in vivo. (A) IP5K pulls down CSN subunits and the cullins. (B) Coexpression of myc-Cul4A and GST-IP5K or its kinase-dead K138A mutant, followed by GST pull-down. (C) Coexpression of GST-IP5K and myc-Cul4A or its various N-terminal truncation mutants, followed by GST pull-down and myc immunoprecipitation. (D) Direct in vitro binding between recombinant IP5K and HEK293-purified Cul4A wild-type or the C-terminal domain ($\Delta 400$). (E) Inositol profiling of cells with shRNA-mediated knockdown of IP5K. (Inset) Western blotting of IP5K. (F) CSN1 immunoprecipitation in control and IP5K knockdown cells. (G) GST pull-down of Cul4A and Cul3 in control and IP5K knockdown cells. (H) IP6 addition to cell lysates increases binding between Cul4A and CSN in IP5K knockdown but not control cells. (I) Bacterial phytase treatment (10 $\mu\text{g}/\text{mL}$, 12 h, 4 $^{\circ}\text{C}$) disrupts the CRL4A–CSN complex.

further supporting IP6 but not its metabolites as a physiologic cofactor in the CRL–CSN complex.

Because both IP5K and IP6K1 interact with Cul4A and metabolize inositol phosphates, we next examined how IP6K1 influences CRL4A–CSN dynamics in the absence of IP5K (21, 32). Consistent with previous data, the IP6K inhibitor TNP increases CRL4A–CSN2 binding in IP5K-proficient cells (Fig. S4G). However, TNP fail to increase CRL4A–CSN2 binding in IP5K knockdown cells (Fig. S4G). Overexpressing IP6K1 in IP5K knockdown cells still rescues the diminished CRL4A–CSN2 interaction, but TNP no longer has any effect (Fig. S4H). Taken together, these data further support our previous conclusion that IP6K1's stabilization of CRL4A–CSN interaction is kinase activity-independent, whereas its role in dissociating CRL4A–CSN complexes is kinase activity-dependent (21), and thus dependent on IP6 generated by IP5K.

IP5K Regulates CRL1/4A Neddylolation and Physiological Function. To understand the role of IP5K/IP6 in cullin physiology, we examined the functional consequences of IP5K knockdown and the resulting loss of CSN binding to cullins. Consistent with CSN being a cullin deneddylase, IP5K knockdown leads to an increased ratio of neddylated to uneddylated Cul1 and Cul4A, without notable effects on Cul2 and Cul3 (Fig. 4A). Similar results are obtained using a different shRNA (Fig. S5A). Total levels of Cul1 and Cul4A are lower in the absence of IP5K (Fig. 4A and Fig. S5A), consistent with the protective role of CSN-associated deubiquitinases in preventing self-ubiquitylation and -destruction of neddylated, active CRLs (33, 34). Indeed, ubiquitylation of Cul1 and Cul4A is augmented in IP5K knockdown cells (Fig. 4B). Moreover, the proteasome inhibitor MG132 and the neddylolation inhibitor MLN4924 (35) both nullify the difference between control and IP5K knockdown cells for both neddylolation/uneddylolation ratio and cullin protein abundance for Cul1/Cul4A (Fig. S5B). These

findings imply that the defects in IP5K knockdown cells reflect altered neddylolation and self-ubiquitylation.

The increased ratio of neddylolated to uneddylolated Cul1/4A with IP5K loss suggests that CRL1 and CRL4A are more active in IP5K knockdown cells. Accordingly, we examined the abundance of cullin substrates. Protein levels of p27 and p21, well-established CRL1 and CRL4A substrates, respectively, are significantly diminished upon IP5K depletion (Fig. 4C), whereas HIF1- α and NRF2, substrates of CRL2 and CRL3, respectively, are unaffected. The altered Cul4A neddylolation ratio and diminished p21 and p27 levels in IP5K knockdown cells are rescued by the expression of shRNA-resistant IP5K, but not its K138A mutant (Fig. 4D). Taken together, these studies indicate that depleting IP6 levels by IP5K knockdown leads to CSN dissociation from CRL1 and CRL4A, resulting in aberrant CRL1 and CRL4A activation.

CRLs target numerous substrates and contribute to a major portion of proteome homeostasis (2). CRL1 and CRL4A, in particular, regulate cell growth and death, with a well-known role for CRL4A in UV-induced apoptosis (2). Accordingly, we examined these traits in cells following IP5K knockdown. Under basal conditions, IP5K knockdown results in pronounced growth defects (Fig. 4E). Moreover, these cells are highly susceptible to UV-induced apoptosis (Fig. 4F). MLN4924-elicited cytotoxicity is also synergized by IP5K depletion (Fig. 4G), consistent with a central role for IP5K/IP6 in regulating CRL1 and CRL4A neddylolation and function.

Discussion

How the assembly of CRL–CSN complexes is dynamically regulated has been enigmatic, which in part explains why few stimuli triggering CRL E3 ligase activity have been identified. Given the mutually exclusive binding of substrate and CSN to substrate receptor (10–12), CSN dissociation and CRL activation may depend

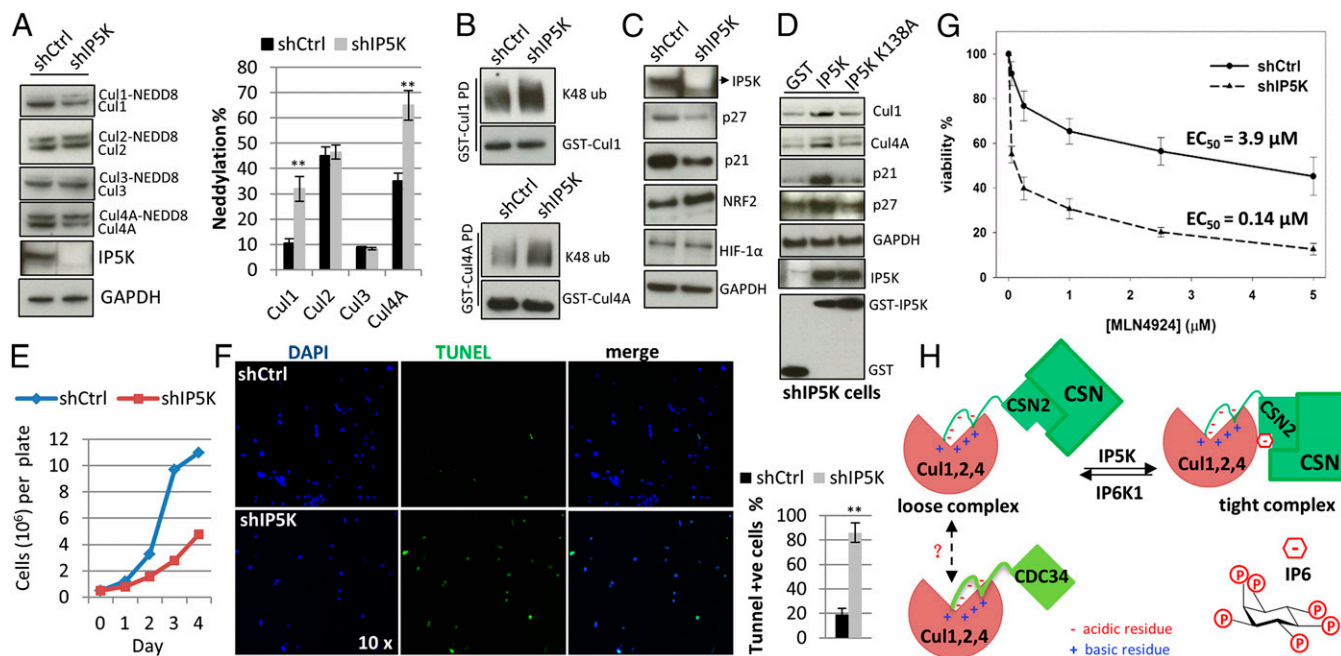


Fig. 4. IP5K regulates Cul1/4A neddylation and function. (A) Levels of neddylated cullins in control and shIP5K cells. Lysis buffer contains 1 mM *N*-ethylmaleimide to minimize deneddylation during processing. (B) Increased levels of K48-linkage-specific polyubiquitination of Cul1 and Cul4A in IP5K knockdown cells. (C) Levels of cullin substrates. (D) Rescue of shIP5K cells with wildtype or kinase-dead IP5K that are resistant to the shRNA because of synonymous mutations. (E) Growth curve of control and shIP5K cells. (F) IP5K depletion exacerbates UV-induced apoptosis, as measured by TUNEL assay. Cells were treated with UV (100 J/m²). After 20 h, cells were stained using the terminal deoxynucleotidyl transferase (TdT)-mediated TUNEL assay system. Fragmented apoptotic cell nuclei were visualized by TUNEL (TdT, green), and the nucleus was stained with DAPI (blue). (G) Viability of control and shIP5K cells after treatment with MLN4924 (0.05, 0.25, 1, 2.5, 5 μM) for 48 h. (H) Scheme depicting the interconversion of cullins between a CSN-bound, inactive state and a CDC34-bound, active state. In this model, CSN2 and E2 ligase CDC34 both bind to cullins' basic canyon via electrostatic interactions (23). IP6 generated by IP5K mediates high-affinity, inactive cullin-CSN complexes, whereas IP6K1 metabolizes IP6 leading to decreased complex affinity (21), which may be followed by cullin activation and CDC34 binding.

on substrate encounter (12). However, the rapid degradation of certain substrates (e.g., CDT1) in response to certain stimuli (e.g., UV light) suggests a complementary, more proactive mechanism. Here, we provide evidence that IP6, with nanomolar potency, participates in CRL-CSN dynamics, particularly for CRL1/4A. The CRLs affect fundamental cellular processes such as cell growth/death and are deregulated in diverse diseases, especially in cancer (3, 36). Targeting IP5K/IP6 and its interface with CRL-CSN in combination therapy may alleviate the dose-limiting toxicity of MLN4924 observed in clinical trials (37).

Prior data have suggested that CSN2 is the subunit of CSN that binds cullins (10, 22). We now demonstrate their direct binding *in vitro*. Moreover, we define the precise structural elements mediating the formation of CRL-CSN complexes. Binding of the acidic tail (amino acids 9–26) of CSN2 to the basic canyon of cullins is reminiscent of interactions between cullins and the E2 ligase CDC34, which also binds to the basic canyon via its C-terminal acidic tail (23). Because the CDC34-bound and CSN-bound forms of cullins represent active and inactive state, respectively, competition between CSN2 and CDC34 could be a novel mechanism whereby CSN inhibits CRL (Fig. 4H).

IP6 is commonly found at protein interaction interfaces, where it usually acts as a “glue” molecule enhancing binding affinity. However, previous studies of IP6's complex-stabilizing role rarely extended to its synthase, IP5K (26, 27, 30). We found that genetic manipulation of IP5K alters CRL1/4A-CSN complex stability, CRL1/4A neddylation status, and downstream CRL1/4A function in a cellular context. By directly binding to the C-terminal domain of cullins, IP5K provides a local pool of IP6 to coordinate CRL-CSN complexes. Prior work has also shown that ITPK1, another enzyme involved in IP6 synthesis, associates with CSN via direct binding to CSN1 (38). Taken together, these data indicate that a local cluster of IPKs may position inositol polyphosphate intermediates for efficient

IP6 generation, analogous to the coupling of classic metabolic enzymes (39).

The role of IP7 in the CRL-CSN complex remains enigmatic. Recently, we reported that, although IP6K1 protein itself stabilizes CRL4A-CSN complexes, the catalytic activity of IP6K1 is required to dissociate CSN from CRL4A *in vivo* (21). *In vitro*, IP7 also promotes CRL4A-CSN2 binding, but with threefold lower potency than IP6 (Fig. 2E). Together with the known instability and low cellular concentration of IP7 relative to IP6, the data strongly suggest that IP7 is not a physiologic “glue,” but rather is behaving differently. IP7 uniquely pyrophosphorylates proteins that have undergone casein kinase 2 (CK2)-mediated phosphorylation (40). CK2 is associated with purified CSN and phosphorylates CSN2 and CSN5 (41). Whether physiologic pyrophosphorylation events underlie IP7's putative involvement in inhibiting CRL-CSN complex formation remains to be examined, pending the development of tools necessary to probe pyrophosphorylation in a cellular context. Moreover, as IP6K1 can also dephosphorylate IP6 to IP5 (42), which is ineffective in assembling CRL-CSN complexes (Fig. S3B), whether the cellular IP6K1 data reflects IP6's turnover to IP7 or IP5 needs to be examined. Nonetheless, our finding that IP6 promotes CRL-CSN complex assembly, whereas its metabolism by IP6K1 disassembles the complex, offers a new perspective on IP6/IP6K biology: namely, IP6Ks catalyze local conversion of IP6 to IP7/IP5 to oppose IP6-mediated effects (Fig. 4H). Consistently, UV-elicited cell death is enhanced by IP5K depletion, but decreased by IP6K1 deletion, possibly because of a differential effect on cullin neddylation; loss of IP5K, but not IP6K1 increases the percentage of neddylated cullins (Fig. 4A) (21). This antagonism between upstream and downstream kinases of IP6 metabolism is also found in regulating the tumor suppressor LKB1. Catalytic activity of IPMK activates LKB1 (43), whereas that of IP6K2 suppresses it (44). IP6 plays structural roles in a number of important cellular

machineries (26, 27, 30, 45), which might also be regulated by one of the three known IP6Ks.

Materials and Methods

Materials. Cell culture, cell counting, and cell death assays (TUNEL and MTT), [³H] inositol profiling, and lentiviral knockdown, Western blotting, real-time PCR, and IP6 imaging by the PAGE method were as described previously (46, 47). The primary antibodies used were: Cul1, Cul2, HIF-1 α , p21, p27 (Santa Cruz); Cul3, Cul4A, CSN5, K48-specific polyubiquitin, and DDB1 (Cell Signaling); GAPDH (Roche); IP5K and CSN2 (ProteinTech); NRF2 (R&D Systems); and GST (Sigma). Polyclonal CSN1 antibody was as described previously (22).

Expression and Purification of Recombinant Protein from *Escherichia coli*. The CSN holo-complex and CSN2 was purified and reconstituted as previously described (32, 48).

In Vitro Binding. GST-cullins were expressed and purified from HEK293 cells using glutathione beads. Every single binding experiment used cullins purified from one 10-cm plate. Endogenous CSN was stripped away by washing in high salt buffer (50 mM Tris-HCl, pH 7.5, 350–400 mM NaCl, 1% Triton X-100). After washing and equilibration with binding buffer [50 mM Tris (pH 7.4), 100 mM NaCl, 0.8% Triton X-100, and 3% (vol/vol) glycerol], the glutathione beads with bound cullins was used for in vitro

binding to purified recombinant CSN2 purified. The EC₅₀ for IP6/7 was determined by fitting the data with the dose–response (stimulation) Equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / [1 + 10^{(\log EC_{50} - x)}]$ (GraphPad). The approximate K_d for CSN2 with/without IP6 was estimated using a one-site binding (with saturation) equation (GraphPad).

Computational Modeling. Protein secondary structure was predicted using the Pspired server (49). Three-dimensional homology models of full-length Cul2 and Cul3 were generated by I-Tasser (50), using Cul4A structure as a template (PDB ID code 2HYE) (24). Images were generated by using Pymol.

Statistical Analysis. All results are presented as the mean and SE of at least three independent experiments. Statistical significance was calculated by Student's *t* test (**P* < 0.05, ***P* < 0.01).

GST pulldown, coimmunoprecipitation, phytase treatment, computational modeling, and a complete list of antibodies and constructs are described in [SI Materials and Methods](#).

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