Coordinated Activation of the Nuclear Ubiquitin Ligase Cul3-SPOP by the Generation of Phosphatidylinositol 5-Phosphate

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Phosphoinositide signaling pathways regulate numerous processes in eukaryotic cells, including migration, proliferation, and survival. The regulatory lipid phosphatidylinositol 4,5-bisphosphate is synthesized by two distinct classes of phosphatidylinositol phosphate kinases (PIPKs), the type I and II PIPKs. Although numerous physiological functions have been identified for type I PIPKs, little is known about the functions and regulation of type II PIPK. Using a yeast two-hybrid screen, we identified an interaction between the type IIβ PIPK isoform (PIPKIib) and SPOP (speckle-type POZ domain protein), a nuclear speckle-associated protein that recruits substrates to Cul3-based ubiquitin ligases. PIPKIIb and SPOP interact and co-localize at nuclear speckles in mammalian cells, and SPOP mediates the ubiquitylation of PIPKIIb by Cul3-based ubiquitin ligases. Additionally, stimulation of the p38 MAPK pathway enhances the ubiquitin ligase activity of Cul3-SPOP toward multiple substrate proteins. Finally, a kinase-dead PIPKIIb mutant enhanced ubiquitylation of Cul3-SPOP substrates. The kinase-dead PIPKIIb mutant increases the cellular content of its substrate lipid phosphatidylinositol 5-phosphate (PI(5)P), suggesting that PI(5)P may stimulate Cul3-SPOP activity through a p38-dependent signaling pathway. Expression of phosphatidylinositol-4,5-bisphosphate 4-phosphatases that generate PI(5)P dramatically stimulated Cul3-SPOP activity and was blocked by the p38 inhibitor SB203580. Taken together, these data define a novel mechanism whereby the phosphoinositide PI(5)P leads to the p38 inhibitor SB203580. Taken together, these data define a novel mechanism whereby the phosphoinositide PI(5)P leads to the p38 inhibitor SB203580. Taken together, these data define a novel mechanism whereby the phosphoinositide PI(5)P leads to the p38 inhibitor SB203580. Taken together, these data define a novel mechanism whereby the phosphoinositide PI(5)P leads to the p38 inhibitor SB203580. Taken together, these data define a novel mechanism whereby the phosphoinositide PI(5)P leads to the p38 inhibitor SB203580.
tors. One of the best known E3 ubiquitin ligases in eukaryotes is the SCF (Skp1/Cul1/F-box) complex, a modular ubiquitin ligase assembled on the cullin protein Cul1. In addition to Cul1, six other cullins (Cul2, -3, -4A, -4B, 5, and -7) have been identified in humans. Cul3-based ubiquitin ligases are an emerging member of this family (13–16). Substrate specificity of Cul3-based ubiquitin ligases is dictated by BTB (Broad complex/Tramtrack/bric-a-brac) domain-containing proteins that bind directly to Cul3 through their BTB domain and bind substrates through a second protein-protein interaction domain (13, 14, 16, 17). Orthologs of Cul3 and BTB proteins have been identified in eukaryotes ranging from Caenorhabditis elegans to humans, and several substrates of Cul3-based ligases have been identified (18–20).

A central theme in the regulation of phosphoinositide signaling pathways is the interaction of enzymes such as PIPKs with upstream regulators and downstream effectors at discrete subcellular sites. To better understand the function and regulation of nuclear phosphoinositide signaling pathways, we sought to identify proteins that interact with PIPKIIβ. Yeast two-hybrid screening identified an interaction between PIPKIIβ and speckle-type POZ domain protein (SPOP), a nuclear speckle-associated BTB domain protein, and substrate adaptor for Cul3-based ubiquitin ligases (13, 14, 16, 17, 19). We demonstrate that PIPKIIβ and SPOP interact in vitro and in vivo and co-localize at nuclear speckles in HeLa cells. We also demonstrate that Cul3-SPOP mediates the ubiquitylation of PIPKIIβ in vivo, and that the ubiquitylation of multiple Cul3-SPOP substrates is potently stimulated by the MKK6-p38 MAPK pathway. Finally, we demonstrate that PI5P, the product of the PI-4,5-P2 4-phosphatases and the lipid substrate of PIPKIIβ, stimulates Cul3-SPOP activity, and this was blocked by the p38 inhibitor SB203580. Taken together, our data support a novel signaling pathway in which PI5P and PIPKIIβ regulate Cul3-SPOP ubiquitin ligase activity through p38 MAPK.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Antibodies**—HEK293 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere with 5% CO2. MG132 (Boston Biochem, Boston, MA), SB203580, and SP600125 (EMD Bioscience, San Diego) were dissolved in Me2SO and added to the media where indicated. Isoxsupropyl 1-thio-β-D-galactopyranoside. Following expression, bacteria were lysed by sonication in PBS containing 0.5% Triton X-100 and Complete mini protease inhibitor mixture (Roche Applied Science). His6 and GST fusion proteins were purified on HisTrap HP and GSTrap FF affinity columns, respectively (Amersham Biosciences), according to the manufacturer’s instructions.

**GST Pulldown and Co-immunoprecipitation Assays**—For GST pulldown assays, equimolar amounts of purified recombinant protein were incubated in 50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, pH 8 in the presence of glutathione-Sepharose resin (Amersham Biosciences). After 4 h at 4 °C, the resin was pelleted at low speed and washed three times in reaction buffer. Bound protein was eluted from the resin by the addition of 2× Laemmli sample buffer, resolved by SDS-PAGE, and detected by Western blot. For co-immunoprecipitation assays, HEK293 cells were transfected via calcium phosphate precipitation by standard methods. 24 h after transfection, cells were washed in PBS and lysed by low amplitude sonication in 50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 0.1% deoxycholate, 0.5 mM EDTA, pH 8. Cell lysates were centrifuged for 15 min at 20,000 × g, and supernatant was recovered. Immunoprecipitations were performed by adding 3 μg of antibody and 20 μl of protein G-Sepharose resin per ml of cell lysate. After incubation at 25 °C for 3 h, the resin was pelleted and washed three times in lysis buffer. Bound protein was eluted in 2× Laemmli sample buffer, resolved by SDS-PAGE, and detected by Western blot.

**Immunofluorescence**—HeLa cells were seeded on glass coverslips and transfected using FuGENE 6 transfection reagent (Roche Applied Science). Twenty four hours after transfection, cells were washed in cold PBS and fixed in methanol. Primary antibodies were diluted to 1 μg/ml in PBS + 0.1% Triton X-100 + 3% bovine serum albumin and incubated on the coverslips at 37 °C. Coverslips were washed three times with PBST and incubated with fluorophore-conjugated secondary antibodies for 1 h at 37 °C. After being washed with PBST, coverslips were mounted onto microscope slides using VectaShield mounting medium (Vector Laboratories). Visualization was performed using a Bio-Rad MRC-1024 laser scanning confocal microscope (W. M. Keck Laboratory for Biological Imaging, Madison, WI).

**In Vivo Ubiquitylation Assays**—HEK293 cells were transiently transfected with the indicated combinations of expression vectors by calcium phosphate precipitation. 24 h after transfection, MG132 was added to the media at a final concentration of 10 μM, and cells were incubated for 6 h at 37 °C. Cells were washed twice with PBS, lysed in PBS with 8 M urea and 0.2% SDS, and sonicated to reduce viscosity. Lysates were incu-
bated for 3 h at 25 °C with nickel-Sepharose resin (Amersham Biosciences). After washing the resin three times with lysis buffer, proteins were eluted in sample buffer, resolved via SDS-PAGE, and detected by Western blot.

RESULTS

The Kinase Insert Domain of PIPKIIβ Is Necessary and Sufficient for Nuclear Targeting—We have demonstrated previously the presence of the type Iα and type Iβ PIP isoforms within mammalian nuclei, including their targeting to nuclear speckles (10). To identify the domain within PIPKIIβ required for its nuclear targeting, a panel of deletion mutants was generated, and their subcellular distributions were assessed by indirect immunofluorescence microscopy. The kinase insert domain of PIPKIIβ was required for nuclear localization, as its deletion prevented nuclear targeting of PIPKIIβ (Fig. 1A). These results are consistent with previous reports that an acidic α-helix within the kinase insert domain promotes nuclear targeting of PIPKIIβ (36, 37). To determine whether the PIPKIIβ kinase insert domain is sufficient for PIPKIIβ nuclear targeting, the kinase insert was fused to LacZ, and its subcellular localization was examined. Whereas a LacZ control was localized entirely within the cytosol, the kinase insert fusion (Ins-LacZ) targeted to the nucleus (Fig. 1B). These results identified the kinase insert domain of PIPKIIβ as being both necessary and sufficient for its nuclear targeting.

Identification of SPOP as a PIPKIIβ-binding Protein—Although the PIPKIIβ kinase insert mediates nuclear translocation, it does not contain a canonical nuclear localization signal. We therefore hypothesized that PIPKIIβ is targeted to the nucleus through its association with interacting partners. To identify proteins that interact with PIPKIIβ, we performed a yeast two-hybrid screen against several human cDNA libraries using the PIPKIIβ kinase insert domain as bait (Fig. 2A). One of the proteins identified by the two-hybrid screen was SPOP (speckle-type POZ domain protein) (Fig. 2A), a nuclear speckle-associated protein and a substrate specificity factor for Cul3-based ubiquitin ligases (18–20).

SPOP Interacts Specifically with PIPKIIβ in Vitro and in Vivo—The interaction between PIPKIIβ and SPOP was assessed using both in vitro and in vivo binding assays. To assess their in vitro association, GST pulldown assays were performed with recombinant purified GST-SPOP and His<sub>6</sub>-PIP<sub>KIIβ</sub>. PIPKIIβ was specifically retained by GST-SPOP but not by GST alone (Fig. 2B), confirming their interaction. SPOP contains two conserved domains: an N-terminal MATH (Meprin and Traf Homology) domain and a C-terminal BTB domain. To assess the contribution of each domain to the interaction between SPOP and PIPKIIβ, GST pulldown assays were performed with purified recombinant GST-MATH and GST-BTB proteins. PIPKIIβ co-precipitated with GST-MATH but not GST-BTB (Fig. 2B), demonstrating that SPOP interacts with PIPKIIβ through its MATH domain. To assess the specificity of SPOP for PIPKIIβ, the highly similar PIPKIIα isoform was also tested. Despite ~80% primary sequence identity between the two PIPKII isoforms, SPOP did not interact with PIPKIIα (Fig. 2C).

The interaction between PIPKIIβ and SPOP was also tested in vivo. HEK293 cells were transiently transfected with mammalian expression constructs of PIPKIIβ and SPOP. Twenty four hours after transfection, PIPKIIβ and SPOP were immunoprecipitated from cell lysates, resolved by SDS-PAGE, and probed by Western blot. PIPKIIβ and SPOP co-precipitated with each other, confirming their in vivo interaction (Fig. 3). Similar to in vitro results, PIPKIIα did not co-precipitate with SPOP. The interaction of endogenous PIPKIIβ and SPOP was also assessed. Endogenous PIPKIIβ was immunoprecipitated from HEK293 lysates using either an α-PIP<sub>KIIβ</sub> antibody or normal rabbit IgG. Western blot analysis identified endogenous SPOP in the PIPKIIβ immunoprecipitates, thus confirming the interaction of endogenous PIPKIIβ and SPOP proteins (Fig. 3C).
Regulation of Cul3-SPOP by PIPKIIβ and Pl-4,5-P_2 4-Phosphatase

**A)** + + - myc-PIPKIIα
- - + myc-PIPKIIβ
+ + + + HA-SPOP

**B)** IP: mAb GA myc mAb GA HA

myc PIPKIIβ

IP: lysate IgG anti-PIPKIIβ

PIPKIIβ

SPop

PIPKIIβ co-localizes with SPOP at Nuclear Speckles—Having confirmed their interaction, we next assessed whether PIPKIIβ and SPOP co-localize within the nucleus. HeLa cells were transiently transfected with SPOP and either myc-PIPKIIβ or myc-PIPKIIα. Twenty four hours after transfection, cells were lysed and immunoprecipitated (IP) with epitope tag-specific antibodies. Normal IgG was used as a negative control. B. endogenous PIPKIIβ was immunoprecipitated from HEK293 cell lysates using an α-PIPKIIβ antibody or normal rabbit IgG. Immunoprecipitates were resolved by SDS-PAGE and probed with α-PIPKIIβ or α-SPOP antibodies. HA, hemagglutinin.

PIPKIIβ and PIPKIIα share ~80% primary sequence identity, with the greatest regions of diversity at the N and C termini and within the kinase insert domain. The specific interaction and co-localization of SPOP with PIPKIIβ therefore suggested that the PIPKIIβ kinase insert is necessary for its co-localization with SPOP at nuclear speckles. To test this hypothesis, chimeric PIPKII mutants were generated in which the kinase insert domains of PIPKIIα and PIPKIIβ were exchanged, and their subcellular distributions were analyzed. PIPKIIα(IIβKI), which contains the PIPKIIβ kinase insert, co-localized with SPOP at nuclear speckles similar to wild type PIPKIIβ. Conversely, PIPKIIβ(IIαKI), which contains the PIPKIIα kinase insert, showed a cytosolic distribution similar to wild type PIPKIIα. These results demonstrate that the kinase insert domain mediates its co-localization with SPOP at nuclear speckles.

SPOP Promotes the Ubiquitylation of PIPKIIβ by Cul3-based Ubiquitin Ligases—The Cul3-SPOP ubiquitin ligase complex has been shown to mediate the ubiquitylation and subsequent degradation of Daxx (19). We therefore assessed whether Cul3-SPOP also promotes the ubiquitylation and degradation of PIPKIIβ. To assess PIPKIIβ turnover, HEK293 cells were transiently transfected with Cul3, SPOP, and the RING-box protein Rbx1, which is required for the activation of cullin-based ligases (21). Endogenous PIPKIIβ levels were assessed by Western blot either 24 or 48 h after transfection. Interestingly, no discernible change in endogenous PIPKIIβ protein levels was detected in transfected cells (data not shown), suggesting that the Cul3-SPOP ubiquitin ligase may not promote PIPKIIβ turnover.

However, as only a fraction of PIPKIIβ (~20%) is nuclear, it would be difficult to detect enhanced turnover of nuclear PIPKIIβ in the context of the total cellular PIPKIIβ.

Next, the ability of Cul3-SPOP to ubiquitylate PIPKIIβ was tested. PIPKIIβ was co-expressed in HEK293 cells with Cul3, Rbx1, SPOP, and His6-ubiquitin. After treating cells with the proteasome inhibitor MG132, cell lysates were purified over nickel resin to capture ubiquitylated proteins (22). Co-expression of the Cul3-SPOP complex promoted ubiquitylation of PIPKIIβ, detected as a ladder of high molecular weight protein by Western blot (Fig. 5A). Efficient ubiquitylation of PIPKIIβ required the Cul3-SPOP complex, as excluding either SPOP or Cul3 and Rbx1 failed to generate ubiquitylated PIPKIIβ. To assess the specificity of the Cul3-SPOP ligase complex for PIPKIIβ, ubiquitylation of PIPKIIβ and PIPKIIα was compared. In contrast to PIPKIIβ, no ubiquitylation of PIPKIIα was observed (Fig. 5B), demonstrating that SPOP specifically promotes ubiquitylation of PIPKIIβ in vivo.

PIPKIIβ Ubiquitylation by Cul3-SPOP Is Stimulated by MKK6/p38 MAPK—p38 MAPK has been shown to phosphorylate PIPKIIβ in response to UV irradiation and oxidative stress, thereby repressing PIPKIIβ lipid kinase activity (12). We hypothesized that p38 might also modulate PIPKIIβ ubiquitylation by Cul3-SPOP.
Regulation of Cul3-SPOP by PIPKIβ and PI-4,5-P\textsubscript{2} 4-Phosphatase

**A**. HEK293 cells were transfected with myc-PIPKII, HA-3x, and HA-SPOP. Ubiquitylation was analyzed for ubiquitylation as in [Fig. 5](#). micro-PIPKII was analyzed for ubiquitylation as in A. C. HEK293 cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, and HA-MKK6+. 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above. D. HEK293 cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, HA-MKK6+, and either myc-PIPKII or myc-PIPKII(S326A). 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above.

**B**. Transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, and HA-MKK6+. 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above. D. HEK293 cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, HA-MKK6+, and either myc-PIPKII or myc-PIPKII(S326A). 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above.

**C**. Transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, and HA-MKK6+. 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above. D. HEK293 cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, HA-MKK6+, and either myc-PIPKII or myc-PIPKII(S326A). 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above.

**D**. Transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, and HA-MKK6+. 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above. D. HEK293 cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, HA-MKK6+, and either myc-PIPKII or myc-PIPKII(S326A). 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above.

**FIGURE 5. SPOP mediates the ubiquitylation of PIPKIβ by Cul3-based ubiquitin ligases.** A, HEK293 cells were transfected with myc-PIPKII, HA-3x, and HA-SPOP. Ubiquitin; immunoblot. 24 h after transfection cells were treated with 10 μM MG132 for 6 h and lysed in the presence of 8 M urea, and ubiquitylated proteins were purified over nickel-Sepharose resin. Ubiquitylated PIPKIβ was detected using α-Myc antibodies. B, HEK293 cells were transfected with the indicated expression constructs. myc-PIPKII and myc-PIPKII were analyzed for ubiquitylation as in A. C, HEK293 cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, and HA-MKK6+. 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above. D, HEK293 cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, HA-MKK6+, and either myc-PIPKII or myc-PIPKII(S326A). 24 h after transfection, cells were treated with MG132, and PIPKIβ ubiquitylation was detected as described above.

p38-dependent attenuation of PIPKIβ activity might be modulated by changes in local concentrations of either PI5P or PI-4,5-P\textsubscript{2}. Previous studies demonstrate that inhibiting PIPKIβ, for example by p38-dependent attenuation of PIPKIβ activity or RNA interference-mediated knockdown of endogenous PIPKIβ, or expression of the kinase-dead PIPKIβ, causes an increase in cellular PI5P levels (11, 12). To test the effect of elevated PI5P levels on PIPKIβ ubiquitylation, the ubiquitylation of wild type PIPKIβ and a well-characterized kinase-dead PIPKIβ point mutant, PIPKIβ(D278A), were compared side-by-side. Ubiquitylation of PIPKIβ(D278A) was dramatically enhanced when compared with the wild type protein (Fig. 6A); furthermore, inhibiting p38 MAPK with SB203580 reduced PIPKIβ(D278A) ubiquitylation (Fig. 6B). These results suggested that increased cellular PI5P levels stimulate PIPKIβ ubiquitylation by Cul3-SPOP, and that this stimulation is transduced by p38 MAPK.

As an independent method to test the hypothesis that PI5P generation stimulates the Cul3-SPOP ubiquitin ligase complex, the ubiquitylation of wild type PIPKIβ by Cul3-SPOP was analyzed by co-expressing either of two recently characterized PI-4,5-P\textsubscript{2} 4-phosphatases (23). Both PI-4,5-P\textsubscript{2} 4-phosphatases caused a strong Cul3-SPOP-dependent increase in PIPKIβ ubiquitylation, similar to the results seen with co-expression of MKK6+ (Fig. 6B). This effect was blocked by SB203580, illustrating a requirement for p38 MAPK downstream of PI5P. Importantly, neither MKK6+ nor the PI-4,5-P\textsubscript{2} 4-phosphatases caused a detectable change in total cellular ubiquitylation (data not shown), reinforcing the specificity of this pathway for ubiquitylation of Cul3-SPOP substrates.

Our observations that PI5P and p38 stimulate the ubiquitylation of PIPKIβ suggested that these signals may cause a general enhancement of Cul3-SPOP activity, thereby promoting the ubiquitylation of numerous Cul3-SPOP substrates. To test this possibility, the effects of PI5P and p38 MAPK on the ubiquitylation of the Fas receptor binding protein Daxx and the pancreatic transcription factor Pdx1 were analyzed. Daxx is a confirmed Cul3-SPOP substrate (19, 24); Pdx1 has not previously been shown to be ubiquitylated by Cul3-SPOP but is a known SPOP-interacting protein, and its transcriptional activity is negatively regulated by SPOP (25, 26). As we observed with PIPKIβ, both Daxx and Pdx1 were ubiquitylated in vivo by Cul3-SPOP, and their ubiquitylation was stimulated by MKK6+ in an SB203580-sensitive manner (Fig. 7A). Furthermore, co-expression of the type I PI-4,5-P\textsubscript{2} 4-phosphatase stimulated the ubiquitylation of both Daxx and Pdx1 (Fig. 7B). Ubiquitylation was attenuated by SB203580, but not by the JNK-specific inhibitor SP600125, reinforcing a specific role for p38 MAPK.
As a complementary approach, the ubiquitylation of Daxx was also assessed in cells overexpressing the kinase-dead PIPKIIβ (D278A) mutant. Kinase-dead PIPKIIβ enhanced the activity of the Cul3-SPOP complex toward Daxx as shown in Fig. 7C. This was also p38 MAPK-dependent, as SB203580 attenuated the effect.

**FIGURE 6. PISP enhances ubiquitylation of PIPKIIβ.** A, HEK293 cells were transfected with FLAG-Cul3, HA-Rbx1, HA-SPOP, and either wild type (WT) or kinase-dead PIPKIIβ (PIPKIIβ[D278A]). 24 h after transfection, cells were treated with MG132 and lysed, and PIPKIIβ ubiquitylation was analyzed. IB, immunoblot. B, ubiquitylation of kinase-dead PIPKIIβ was analyzed as in A in the absence or presence of 2 μM SB203580. C, HEK293 cells were transfected with the indicated combinations of myc-PIPKIIβ, FLAG-Cul3, HA-Rbx1, HA-SPOP, and FLAG-tagged type I or type II PI-4,5-P2 4-phosphatase. PIPKIIβ ubiquitylation was analyzed 24 h after transfection.

**FIGURE 7. PISP stimulates Cul3-SPOP activity toward multiple substrates.** A, myc-Daxx or myc-Pdx1 were expressed in HEK293 cells with the indicated combinations of FLAG-Cul3, HA-Rbx1, HA-SPOP, and HA-MKK6+. Ubiquitylation of Daxx and Cul3 was analyzed 24 h after transfection. IB, immunoblot. B, HEK293 cells were transfected with FLAG-Cul3, HA-Rbx1, HA-SPOP, FLAG-type I PI-4,5-P2 4-phosphatase, and either myc-Daxx or myc-Pdx1. Where indicated, transfected cells were treated with SB203580 or SP600125. Ubiquitylation of Daxx and Pdx1 was analyzed 24 h after transfection. C, HEK cells were transfected with the indicated combinations of FLAG-Cul3, HA-Rbx1, myc-Daxx, and either HA-MKK6+, FLAG-type I PI-4,5-P2 4-phosphatase, or kinase-dead FLAG-PIPKIIβ. Where indicated, cells were treated with SB203580. Ubiquitylation of Daxx was assessed 24 h after transfection.

As a complementary approach, the ubiquitylation of Daxx was also assessed in cells overexpressing the kinase-dead PIPKIIβ(D278A) mutant. Kinase-dead PIPKIIβ enhanced the activity of the Cul3-SPOP complex toward Daxx as shown in Fig. 7C. This was also p38 MAPK-dependent, as SB203580 attenuated the effect.

**SPOP Is Also Ubiquitylated Downstream of PISP and MKK6—** Several F-box and SOCS proteins have been shown previously to be degraded by Cul1 and Cul2 ubiquitin ligases, respectively (27–29). The *C. elegans* BTB protein MEL-26 also appears to be degraded by Cul3 ligases (14, 15), suggesting a general mechanism whereby substrate adaptor proteins like SPOP are
Regulation of Cul3-SPOP by PIPKIIβ and PI-4,5-P2 4-Phosphatase

Figure 8. SPOP is ubiquitylated by Cul3 downstream of p38 MAPK. A, diagrams of wild type (WT) SPOP and SPOP truncation mutants assayed for ubiquitylation. B, HEK293 cells were transiently transfected with His6-ubiquitin and the indicated SPOP expression vectors. 24 h after transfection, SPOP was analyzed for ubiquitylation as described above. IB, immunoblot. C, HEK293 cells were transfected with myc-SPOP and the indicated combinations of FLAG-Cul3, HA-Rbx1, and HA-MKK6+. SPOP ubiquitylation was analyzed 24 h after transfection.

PI5P modulates an upstream activator of p38 MAPK, resulting in the general inactivation of the MKK6+ pathway. Together, these results define a novel mechanism whereby signaling proteins are ubiquitylated by multiple Cul3-SPOP substrate proteins, including the SPOP-binding proteins Daxx and Pdx1. These data demonstrate that p38 stimulates the ubiquitylation of multiple Cul3-SPOP substrate proteins, including Daxx and Pdx1. These data suggest that PI5P functions in vivo by activating the p38 MAPK pathway further.

Figure 9. Model of PI5P- and p38-sensitive regulation of Cul3-SPOP activity. Under resting conditions, PI5P maintains low PI3P levels by synthesis of PI-4,5-P2. In response to a stimulus such as UV irradiation or oxidative stress, PI5P is attenuated, resulting in accumulation of PI-4,5-P2, stimulating Cul3-SPOP activity toward multiple substrates, including Daxx and Pdx1.
Cul3-SPOP ubiquitin ligase activity, although currently the mechanism by which this occurs is unknown. One possibility is that either p38 or a p38 effector kinase phosphorylates Cul3-SPOP, thereby stimulating its catalytic activity. In support of this hypothesis, p38 has been reported to phosphorylate the RING E3 ubiquitin ligase Siah2, increasing its activity toward its substrate PHD3 (30). Further characterization is required to determine how p38 activation results in stimulation of Cul3-SPOP activity.

A key discovery from our experiments is the ability of PI5P to stimulate Cul3-SPOP activity through a p38-dependent signaling pathway. Although relatively little about the physiological functions of PIPKII is currently known, one recurring theme is the role of its substrate, PI5P, as a key regulatory molecule. Previous reports have linked PIPKII to the role of its substrate, PI5P, as a key regulatory molecule. Our results now identify a third signaling pathway in the cell. This pathway is primarily regulated by PI5P and stimulates Cul3-SPOP.

The mechanism by which PI5P activates p38 MAPK is also critical. We have shown that PI5P activates p38 MAPK through a p38-dependent signal transduction pathway. This pathway would be more distal to the E2 enzyme and therefore a less efficient target for ubiquitination. Other Cul3 specificity factors may be similarly ubiquitylated within their substrate-binding domains. A more detailed analysis would be beneficial to more thoroughly understand the function of Cul3 specificity factors as well as the activities of Culin-based ubiquitin ligases as a whole.

In summary, the data presented in this study define a novel mechanism by which phosphoinositide signaling regulates a nuclear ubiquitin ligase complex. The interaction of PIPKII with the Cul3-SPOP ubiquitin ligase, coupled with the ability of the PI5P substrate PI5P to modulate Cul3-SPOP activity through a signaling pathway inhibited by the p38 inhibitor SB203580, identifies a new function for phosphoinositide signaling within the nucleus. Identification of stimuli that enhance or attenuate this pathway, as well as delineation of the downstream physiological effects, will be invaluable to more thoroughly understand the roles of phosphoinositides within the nucleus.

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