**Significance**

The 20S proteasome is a key actor of the control of protein levels and integrity in cells. To perform its multiple functions, it works with a series of regulators, among which is a nuclear complex called PA28γ. In particular, PA28γ participates in the regulation of cell proliferation and nuclear dynamics. We describe here the characterization of a protein, PIP30/FAM192A, which binds tightly to PA28γ and favors its interaction with the 20S proteasome while inhibiting its association with coilin, a central component of nuclear Cajal bodies. Thus, PIP30/FAM192A critically controls the interactome and, consequently, the functions of PA28γ, and appears to be a previously unidentified player in the fine regulation of intracellular proteostasis in the cell nucleus.


The authors declare no conflict of interest.

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**PIP30/FAM192A is a novel regulator of the nuclear proteasome activator PA28γ**


PA28γ is a nuclear activator of the 20S proteasome involved in the regulation of several essential cellular processes, such as cell proliferation, apoptosis, nuclear dynamics, and cellular stress response. Unlike the 19S regulatory subunit of the proteasome, which specifically recognizes ubiquitylated proteins, PA28γ promotes the degradation of several substrates by the proteasome in an ATP- and ubiquitin-independent manner. However, its exact mechanisms of action are unclear and likely involve additional partners that remain to be identified. Here we report the identification of a cofactor of PA28γ, PIP30/FAM192A. PIP30 binds directly and specifically via its C-terminal end and in an interaction stabilized by casein kinase 2 phosphorylation to PA28γ. Its recruitment to proteasome-containing complexes depends on PA28γ and its expression increases the association of PA28γ with the 20S proteasome in cells. Further dissection of its possible roles shows that PIP30 alters PA28γ-dependent activation of peptide degradation by the 20S proteasome in vitro and negatively controls in cells the presence of PA28γ in Cajal bodies by inhibition of its association with the key Cajal body component coilin. Taken together, our data show that PIP30 deeply affects PA28γ interactions with cellular proteins, including the 20S proteasome, demonstrating that it is an important regulator of PA28γ in cells and thus a new player in the control of the multiple functions of the proteasome within the nucleus.
diffuse through the 20S pores, and therefore PA28γ stimulation of their degradation can be explained by 20S-pore opening only. However, how PA28 complexes recruit protein substrates and deliver them to the proteasome is not understood, as these complexes are a priori inert molecules that, unlike the 19S complex, do not possess any ATPase activity that could provide energy and movement to unfold the substrates and inject them into the 20S proteasome (2, 3). A likely possibility is thus that PA28 complexes function in proteasome-dependent proteolysis in association with other proteins that remain to be defined.

In this study, we describe a partner of a PA28 complex, the evolutionary conserved PIP30/FAM192A protein. We show that PIP30 binds with high specificity to PA28γ and enhances its association with the 20S proteasome in cells. Importantly, PIP30 binding affects PA28γ specificity toward peptide substrates in vitro as well as its interactions with cellular proteins, such as the CB marker coilin. Therefore, PIP30 is a major regulator of PA28γ functions and consequently of the nuclear functions of the proteasome.

Experimental Procedures

Phylogenetic Analyses. Genomes were explored by using Annotation and BLAST search tools available in the Geneious 9.1.7 software package (www.geneious.com) and amino acid sequences were aligned (MAFFT v7.017) and a phylogenetic tree was deduced by maximum-likelihood analysis (PhyML).

Antibodies. Antibodies and related agents used in this study are described in SI Appendix, Material and Methods.

Production of 20S Proteasome and Recombinant Proteins. Native 20S proteasome was purified from extracts of HeLa cells (Iscracell) using classic chromatographic procedures (26). Unless indicated, recombinant PA28γ was produced in Escherichia coli BL21 DE3 CodonPlus as a 6His-tagged protein and purified by affinity purification followed by proteolytic removal of the tag, anion-exchange chromatography, and gel filtration (SI Appendix, Fig. S10B). At the last step, the protein was eluted slightly earlier than the BSA (67 kDa) marker (i.e., at an apparent molecular weight larger than twice what was expected). Human recombinant PA28γ was produced and purified from E. coli, as previously described (27). Human PA28γ was expressed in E. coli BL21 DE3 CodonPlus. After expression, the PA28γ complex was purified by chromatography as PA28βg, with some minor modifications of the procedure. Both PA28βg and PA28γ complexes efficiently activated the peptide activities of the 20S proteasome. Recombinant GST and GST-PIP30-H201 fusion proteins were produced in bacteria and efficiently purified using glutathione Sepharose beads.

Pull-Down and Immunoprecipitation. For immunoprecipitation (IP) of GFP-fusion proteins, U2OS cells were transfected with the indicated constructs. Twenty-four hours posttransfection, cells were homogenized in lysis buffer [25 mM Heps pH 7.8, 100 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 1% IGEPALECA-630, 1.0% Triton X-100, 1 mM DTT, 1 mM ATP, 10% glycerol (vol/vol)], in the presence of complete EDTA-free protease inhibitor mixture (Roche), for 15 min on ice. After centrifugation at 15,000 × g for 15 min (4 °C), supernatants were recovered and protein concentration was determined by Bradford assays using BSA as a standard. Next, 20 μL of GFP-TRAP-A beads (Chromotek) were used per IP, mixed with 200 μg of protein extract, and incubated with constant gentle stirring for 1 h at 4 °C. Beads were washed three times with lysis buffer and boiled in 2× Laemmli sample buffer. Samples were then analyzed by SDS/PAGE and immunoblotting.

Endogenous PIP30 and PA28γ were immunoprecipitated from total cell extracts using anti-PA28γ (rabbit) and anti-PA28β (rabbit) antibodies, bound to protein A-Sepharose (GE Healthcare) and, after blocking, incubated with 1 μg of HA-antibody (Roche) diluted 1:500 in blocking solution for 1 h at 4 °C.

For co-IP of colin and PA28γ proteins (see, for example, Fig. S5B), nuclear extracts were prepared as described in ref. 21. Briefly, U2OS and PIP30−/− cell pellets were resuspended in hypotonic buffer (10 mM Tris-HCl pH 7.5, 10% glycerol, 4 mM DTT, 50 mM NaF, 1 mM NaVO₃, 1 mM MgCl₂) in the presence of complete EDTA-free protease inhibitor mixture (Roche). IGEPALECA-630 was then added at the final concentration of 0.5% and cells were incubated on ice for 3 min. After centrifugation at 800 × g for 5 min, the pellets, protein A-Sepharose beads bound to HA-antibody, were resuspended in digestion buffer (2 mM Tris-HCl pH 8.5, 20% glycerol, 10 mM DTT, 50 mM NaF, 1 mM NaVO₃, 1 mM MgCl₂, 5 mM CaCl₂, 1× complete protease inhibitor mixture supplemented with 75 μM micrococal nuclease), and then digested for 15 min at 25 °C with constant stirring. At the end, an equivalent volume of extraction buffer (2 mM Tris-HCl pH 8.5, 50 mM NaF, 1 mM NaVO₃, 1 mM MgCl₂, 20 mM EDTA, 0.84 M KCl, 1× complete protease inhibitor mixture) was added and the mix was incubated on ice for 20 min. Nuclear extracts were clarified by centrifugation for 30 min at 15,000 × g. Before IP, KCI concentration was reduced to 280 mM, 3 μg of anticolin or control IgG were added to 400 μg of nuclear extract and incubated for 2 h at 4 °C. IP proteins were collected by addition of 15 μl of protein A-Sepharose beads. After extensive washes, beads were boiled in 2× Laemmli sample buffer and samples were analyzed by SDS/PAGE and immunoblotting.

Mass Spectrometry Analyses. Stable isotope labeling by amino acids in cell culture (SILAC) IPs (endogenous PA28γ and GFP-FAM192A/PIP30) were essentially performed as previously described in ref. 28. Further details are provided in SI Appendix.

Whole human proteasome complexes, including 20S-bound activators and regulators, were immunopurified and analyzed by quantitative mass spectrometry, as previously described (29, 30). Next, 2 × 10⁵ in vivo formaldehyde-cross-linked human cells (HeLa and U937, three biological replicates per cell line) were used. For complete nuclear proteasome interactome analysis, U937 cells nuclei were prepared and, before proteasome purification, the purity of nuclear proteasomes was assessed both by Western blot and MS analysis, as detailed earlier (31). Purified proteasome complexes were analyzed by mass spectrometry as previously described (32). Further details are provided in SI Appendix section.

Native Electrophoresis in Tris-Glycine System. Recombinant protein samples were incubated 5–10 min at room temperature in reaction buffer (Tris-HCl 20 mM, pH 7.5, DTT 1 mM, Glyceral 10% (vol/vol)), then supplemented with 1 μl of native sample buffer (xylenyl cyanol FF in reaction buffer supplemented with 50% glycerol) and applied on 5% polyacrylamide gel prepared in Tris-Glycine electrophoresis buffer (25 mM Tris-HCl pH 8.0, 192 mM Glycine, 1 mM DTT). Native electrophoresis was performed for 4.5–5 h (100 V, 4 °C). After denaturation in 10x TG-SDS buffer, proteins were transferred on PVDF membrane and immunoblotted.

Surface Plasmon Resonance Analysis. Experiments were performed on Bioanalyzer 3000 apparatus (GE Healthcare) at 25 °C using a flow rate of 50 μl/min in HBS-EP buffer (GE Healthcare). To compare the binding of PA28γ and PA28βg on 6His-PIP30 recombinant protein, 6His-PIP30 (AS50R)U was captured on anti–His-Tag covalently immobilized on a CM5 sensor chip using an amine coupling procedure according to the manufacturer’s instructions. A control flowcell was obtained with the same chemical procedure without protein. Then, 60 μl of PA28γ and PA28βg (50 μg/ml) were injected on His6PIP30 and control flowcells followed by a dissociation step of 400 s.

In Vitro CK2 Phosphorylation Assay. GST and GST-PIP30-H201 proteins were incubated with recombinant CK2 according to the manufacturer’s instructions. For radioactive kinase assays, 32P labeled ATP (1 μCi in the presence of 100 μM cold ATP) was added. Reactions were stopped either by adding 5 mM EDTA or Laemmli sample buffer.

Proteasome Peptide Assays. Peptidase activities of 20S proteasome were measured using black flat-bottom 96-well plates (Nunc), in a final volume of 50 μl, in reaction buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 10% glycerol (vol/vol) supplemented with 100 μM peptide substrates. When indicated, purified recombinant PA28β and/or PA28γ were added. Kinetic analyses showed that the assays are linear at least for 30 min. Peptide degradation was measured by the fluorescence emitted by the AMC group released by cleavage of the substrate (excitation 380 nm, emission 440 nm) using a FLX800 microplate fluorescence reader (Bio-Tek Instruments).

Immunofluorescence Microscopy and Proximity Ligation Assays. Cells were fixed in 3.7% paraformaldehyde/PBS for 10 min at room temperature, washed with 0.1% Triton X-100 for 1 min at room temperature. Coverslips were then blocked in blocking solution (1% FCS, 0.01% Tween-20/PBS) for 10–20 min and incubated with primary antibodies, diluted in blocking buffer, for 1 h at room temperature or 37 °C in a humidified atmosphere. After three washes in PBS, coverslips were incubated with Alexa-Fluor conjugated secondary antibodies diluted in blocking solution for 40 min at room temperature. Coverslips were washed with PBS, incubated with 0.1 μg/ml DAPI solution in PBS for 5 min at room temperature, washed twice in PBS, and finally once in H₂O. Coverslips were mounted on glass slides using ProLong Gold antifade reagent (Thermo Fisher Scientific).

For proximity ligation assays (PLA), cells on coverslips were fixed and permeabilized as described in ref. 22. Coverslips were then blocked in a
solution provided by the Duolink kit. Cells were then incubated with m
tioned antibodies as described above. Duolink In Situ PLA Probe Anti-Rabbit MINUS and Anti-Mouse PLUS and Duolink In Situ Detection Reagents (Sigma-Aldrich) were used, according to the manufacturer’s instructions.

Image Acquisition and Analysis. The z-stacks and images were acquired with a 63×/1.4 NA or 40× oil-immersion objective lenses using widefield microscopes, DM6000 (Leica Microsystems) or Axiosimager Z1 or Z2 (Carl Zeiss), equipped with coolSNAP HQ2 cameras (Photometrics). Images were acquired as TIF files using MetaMorph imaging software (Molecular Devices). For CB and PLA dot quantitative analysis, z-stacks were acquired every 0.3–0.4 μm (z-step) with a range of 10–15 μm to image the whole nuclei. The number of PLA foci was analyzed with ImageJ. The size, the intensity, and the number of CBs were measured with ImageJ (1.49v), using a specific “macro” that has been created to automatically quantify these different parameters. The script allows creating a mask of DAPI image to isolate the nucleus of each cell and create a maximum-intensity projection of the 25 z-stacks. The mask is used in the maximum-intensity projection to count the number of CB or PLA dots of each nucleus via an appropriate thresholding. The “Analyze Particles” tool of ImageJ was used to calculate the size and the mean gray value of each CB.

Results

FAM192A Is a Partner of both Free and 20S Proteasome-Bound PA28γ That Regulates the Interaction Between PA28γ and 20S Proteasome. To identify new interactors of PA28γ, we used a high-throughput approach combining endogenous PA28γ IP and SILAC-based quantitative proteomics (28) (SI Appendix, Fig. S1A, Left). In this experiment, 68 human protein groups containing at least two unique peptides were quantified and visualized by plotting log2(heavy/light) (H/L) ratio versus log2(H intensity) (Fig. L4 and Dataset S1). PA28γ itself was found with a H/L SILAC ratio close to 15. Among the PA28γ interactors was FAM192A, a poorly annotated nuclear protein of unknown cellular function, whose expression is induced during skeletal muscle atrophy (33). FAM192A was also identified with a high SILAC ratio in a GFP-USP14, were found in this category [abundance ratios (IP 20S/IP minus 20S)] (SI Appendix, Fig. S3) demonstrated that FAM192A is also recruited on hybrid proteasomes, most likely via its interaction with PA28γ.

Interestingly, these experiments also showed that co-IP of PA28γ with 20S proteasome was halved (P = 0.02) in siRNA FAM192A-depleted cells (Fig. 1D). This result was confirmed by reverse anti-PA28γ co-IP experiments using wild-type and FAM192A−/− U2OS cell extracts, showing that indeed FAM192A depletion elicits a twofold decrease in PA28γ/20S interaction (Fig. 1E). Thus, FAM192A promotes the association of PA28γ with the 20S proteasome.

Altogether, our experiments establish that FAM192A is a major direct interactor of PA28γ, recruited to proteasome complexes via PA28γ and favoring the association of PA28γ with the 20S proteasome. FAM192A is also called NIP30 (NEFA-interacting protein 30 kD, which might refer to a NIP-4-hydroxy-5-iodo-3-nitrophenyl acetate) labeled 30-kD protein that interacts with phosphatidylinositol biphosphate (34). Based on the above experiments, and since FAM192A has no clear assigned cellular function, we propose to rename it PIP30, for PA28γ interacting protein 30 kDa. This name will be used thereafter in this report.

PA28γ/IP30 Interaction Is Stabilized by CK2 Phosphorylation of IP30 C Terminus. Evolutionary analyses show that the IP30 sequence is characterized by the presence of three conserved domains: an N-terminal signature domain (pfam10187, also called NIP30 domain, ~100 amino acids) and two smaller motifs (SI Appendix, Fig. S3A). The pfam10187 domain is found in metazoa as well as in all major eukaryotic supergroups. This allows tracing back the IP30 gene to early stages of eukaryote evolution (SI Appendix, Fig. S3B). As it is the case for PA28γ (4), the conserved C-terminal motifs of IP30 proteins are enriched in phosphatidylinositol residues, including a completely conserved tyrosine and several serine residues (SI Appendix, Fig. S3A).

Using various truncation mutants, we determined that the last 54 amino acids of IP30 (amino acids 201–254; i.e., the H201 mutant) are necessary and sufficient for interaction with endogenous PA28γ and that amino acids 223–230 are critical for the association (Fig. 2A and SI Appendix, Fig. S4A and B). This C-terminal region includes the highly conserved serine-rich and acidic sequence highlighted in SI Appendix, Fig. S3A and contains two SDSE motifs (SI Appendix, Fig. S4C) that both match the canonical consensus site (S/T-X-X/E/D/pS/pT) for casein kinase 2 (CK2) (35, 36). Indeed, we found that this region can be phosphorylated by CK2 in vitro (Fig. 2B). Phosphorylation is dependent on the integrity of the CK2 consensus sites, as it was reduced when the serine residues S222 and S228 were replaced by alanine (mutant SS-AA), and abolished or strongly impaired when the acidic residues within the two CK2 consensus motifs were converted into basic lysine residues (mutants D222E225, KK and D225G228,R229,KK, respectively) (Fig. 2B).

In cells, in the context of the full-length GFP-PIP30 protein (Fig. 2C and SI Appendix, Fig. S4D), like in vitro with the C-terminal part of PIP30 (SI Appendix, Fig. S4E), these mutations altered the binding to PA28γ. In a manner parallel to their effects on CK2 phosphorylation in vitro, the acidic mutants abolished or strongly impaired the binding to PA28γ, while the SS-AA mutant reduced it (Fig. 2C and SI Appendix, Fig. S4E). This weaker binding of the SS-AA mutant strongly suggests that phosphorylation is stabilizing PA28γ/IP30 interaction. Indeed, when using purified proteins, PA28γ showed more affinity for CK2-phosphorylated than for non-phosphorylated PIP30 (Fig. 2D).

To verify that CK2 is the endogenous kinase phosphorylating PIP30, we treated cells with the selective CK2 inhibitor CX-4945 (37). This treatment revealed a second, faster-migrating band (Fig. 2E), suggesting that in cells PIP30 is primarily in a CK2-phosphorylated form. This conclusion is strengthened by thorough
analysis of the phosphorylated semitryptic PIP30 peptides found in our mass spectrometry analyses of anti-PIP30 immunoprecipitates (SI Appendix, Fig. S5). While only a minor fraction of endogenous PIP30 was nonphosphorylated in wild-type cells upon CK2 inhibition for 24 h, it was mainly non- or hypophosphorylated in PA28γ−/− cells after the same treatment (Fig. 2E). This finding suggests that, although PIP30 does not require PA28γ for being phosphorylated, the binding to PA28γ protects it from being dephosphorylated. This was confirmed by the fact that λ-phosphatase can dephosphorylate a fraction of immunoprecipitated endogenous PIP30, but not the endogenous PIP30 coimmunoprecipitated with PA28γ (SI Appendix, Fig. S4F). Finally, after CX-4945 treatment, only the phosphorylated PIP30 was retrieved upon PA28γ IP (Fig. 2F), showing that the phosphorylation of PIP30 by CK2 in cells stabilizes its interaction with PA28γ. Taken together, our results show that the C-terminal end of PIP30 protein is critical for its binding to PA28γ and that its phosphorylation by CK2 stabilizes this interaction.

**PIP30 Controls Substrate Diffusion to the Catalytic Chamber of the Proteasome.** We next assessed whether PIP30 could interfere with the best-known property of PA28γ: that is, its ability to activate the peptidase activities of the proteasome in vitro. Using recombinant PIP30 in combination with PA28γ and the 20S proteasome, we found that PIP30 differentially altered
degradation of a panel of standard proteasome peptide substrates (38) by the PA28γ-activated proteasome (Fig. 3A). For example, while the activation of Suc-LLVY-amc (LLVY) and Ac-nLPnLD-amc (nLPnLD) degradation was partially inhibited, degradation of Boc-LRR-amc (LRR) was essentially insensitive to the presence of PIP30 for the indicated times. The presence of GST-tagged proteins was revealed by Ponceau staining and their phosphorylation was detected by autoradiography. (C) Point mutations in the CK2 consensus motifs of GFP-PIP30 strongly reduce the interaction between GFP-PIP30 and PA28γ in cells. U2OS cells were transiently transfected with wild-type or the indicated mutants of PIP30 fused to GFP. GFP-tagged proteins were pulled down using GFP-TRAP beads and the presence of coimmunoprecipitated PA28γ was assessed by Western blot. Wild-type GFP-PIP30 was used as a positive control and GFP-PIP30-199 mutant as a negative control. (D) PA28γ has more affinity for CK2-phosphorylated PIP30 (PIP30p) than for unphosphorylated PIP30 in vitro. Bacterially expressed and purified His-PIP30 was either phosphorylated, or not, by CK2 in vitro and then mixed with recombinant PA28γ. The complex was purified on a Ni-NTA column and analyzed by SDS/PAGE and Western blot with anti-His anti-PA28γ antibodies. (E) The association between PA28γ and PIP30 protects PIP30 from dephosphorylation. Wild-type and PA28γ−/− U2OS cells were either treated, or not, with CX-4945 (10 μM) for 24 h and the PIP30 PAGE migration profile was analyzed by Western blot. The phosphorylated form (PIP30p) and the unphosphorylated form of PIP30 are indicated. (F) PA28γ interacts with the phosphorylated form of endogenous PIP30 in cells. Endogenous PA28γ was immunoprecipitated from 500 μg of total extracts from U2OS cells either treated, or not, with CX-4945 (10 μM) for 24 h. The presence of coimmunoprecipitated PIP30 was assessed by Western blot. The input and unbound (SN) fractions were also analyzed. After CX-4945 treatment, two bands appear for endogenous PIP30, the phosphorylated form (PIP30p) and the unphosphorylated form (PIP30), as indicated. CTL, untransfected cells.
upon binding to the 20S proteasome and PA28γ, do not rescue the phenotype, compared with GFP alone (Fig. 4D). Furthermore, the PIP30 mutants impaired in their binding to PA28γ (i.e., GFP-PIP30γKK, D225E225-KK, and D229E229-KK) did not significantly rescue the phenotype, compared with GFP alone (Fig. 4D). Together, these results show that PIP30, like PA28γ (21), controls the steady-state number of CBs and that this depends on its binding to PA28γ. However, PIP30 and PA28γ do not have the same effects in this process. An important question was to assess whether the effect of PIP30 depletion is dependent upon the presence of cells in PA28γ. We thus compared the effect of PIP30 knockdown in wild-type and PA28γ−/− cells (Fig. 4E). The results show that in both cell lines depletion of PIP30 entails a decrease in the number of cells with CBs. However, the effect of PIP30 depletion seems less drastic in PA28γ−/− compared with wild-type cells. Together, these results suggest two components in the functions of PIP30 in CB dynamics: one critical that is PA28γ-dependent, and another that is PA28γ-independent.

In the absence of PIP30 (i.e., in siRNA PIP30-depleted or PIP30 knockout cells, but not in control cells) we observed an accumulation of PA28γ in all residual CBs, either identified by coilin (Fig. S4) or WRAP53 (another CB marker) staining (SI Appendix, Fig. S7A). Furthermore, reexpression of GFP-PIP30 in PIP30-depleted cells abrogated the accumulation of PA28γ in CBs (SI Appendix, Fig. S7B). These observations demonstrate that PIP30 inhibits PA28γ subnuclear localization in CBs.

**PIP30 Alters PA28γ Functions in CB Dynamics.** We have previously shown that PA28γ overexpression leads to the disruption of CBs (21), which are evolutionary conserved colin-dependent and colin-rich subnuclear compartments. CBs are involved in the maturation of small nuclear ribonucleoproteins (RNPs) and small nucleolar RNPs, as well as in the processing of histone mRNAs (41, 42). We thus tested whether PIP30 has also a role in CB dynamics. First, we compared CB number in wild-type, PA28γ−/−, and PIP30−/− U2OS cells. We found that while 80% of wild-type and PA28γ−/− U2OS cells display CBs, only 40% of PIP30−/− cells are CB+ (Fig. 4A and B). Furthermore, in cells displaying CBs, the absence of PIP30 leads to a decrease in the average number of CBs per nucleus (Fig. 4C). The effect of PIP30 depletion was rescued by expression of GFP-PIP30 (Fig. 4D). Furthermore, the PIP30 mutants impaired in their binding to PA28γ (i.e., GFP-PIP30γKK, D225E225-KK, and D229E229-KK) did not significantly rescue the phenotype, compared with GFP alone (Fig. 4D). Together, these results show that PIP30, like PA28γ (21), controls the steady-state number of CBs and that this depends on its binding to PA28γ. However, PIP30 and PA28γ do not have the same effects in this process. An important question was to assess whether the effect of PIP30 depletion is dependent upon the presence of cells in PA28γ. We thus compared the effect of PIP30 knockdown in wild-type and PA28γ−/− cells (Fig. 4E). The results show that in both cell lines depletion of PIP30 entails a decrease in the number of cells with CBs. However, the effect of PIP30 depletion seems less drastic in PA28γ−/− compared wild-type cells. Together, these results suggest two components in the functions of PIP30 in CB dynamics: one critical that is PA28γ-dependent, and another that is PA28γ-independent.

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**Fig. 3.** PIP30 differentially affects peptide diffusion toward the catalytic chamber of the proteasome. (A) Inhibition by PIP30 of PA28γ-activated 20S proteasome is peptide substrate-specific. Proteasome activity was assayed in the presence of the indicated peptides and proteins and normalized by setting to 100% the value obtained in the presence of 20S proteasome and PA28γ. The assays were performed for 20 min at 37 °C in a 50-μL reaction mixture containing the indicated combinations of 20S proteasome (0.5 μg), PA28γ (1 μg), and PIP30 (4 μg). The respective final concentrations of the 20S proteasome and PA28γ are set up such that the peptide activities are not fully activated (i.e., PA28γ is limited in the assay). At the final concentration used for PIP30, the inhibitory effect is maximal (SI Appendix, Fig. S6A). Error bars represent deviation from the mean of technical duplicates. The figure is representative of three distinct experiments. C-L, caspase-like; CT-L, chymotrypsin-like; T-L, trypsin-like.

**Discussion.**

The results indicate that PIP30 inhibits PA28γ-dependent but not PA28γ-independent functions in CBs. The inhibitory effect of PIP30 is maximal when the phosphorylation status of the target substrate is not modified by PIP30: 20S proteasome (2 μg) was mixed in reaction buffer (Tris HCl 20 mM pH 7.5, DTT 1 mM, glycerol 10%) in a final volume of 18 μL, with either no protein or with PA28γ (6 μg) preincubated for 5 min at room temperature with or without PIP30 (8.3 μg). After incubation 5 min at room temperature, 2 μL of the probe Bodipy TMR-Ahx-LVS (1 μM in reaction buffer) were added to each sample and the mixtures were further incubated 15 min at 37 °C. After denaturation with Laemmli buffer and electrophoresis, the labeled peptide subunits were visualized (Left) using a Typhoon FLA 7000 (GE Healthcare). The labeling of subunits β2 and β5 was quantified and plotted after normalization by the value of their labeling in 20S proteasome alone (Right). In the experimental conditions used, the labeling of both subunits is roughly proportional to their activity. The figure is representative of three distinct experiments. (C) Comparison of the effects of PIP30 and its CK2-phosphorylated form (PIP30-PH) on the peptide activities of the PA28γ/20S proteasome complex. The experiments were performed as in A, except that only 2 μg of PIP30 and PIP30-PH were used per assay. Error bars represent deviation from the mean of technical duplicates. The figure is representative of three distinct experiments. C-L, caspase-like; CT-L, chymotrypsin-like; T-L, trypsin-like.
Because PA28γ is known to bind to the key CB component coilin (21) (Dataset S2), we analyzed whether PIP30 could affect this process. We observed that PIP30 depletion led to an increased interaction of PA28γ with coilin, as seen by co-IP experiments (Fig. 5B). In line with this observation, in the absence of PIP30, an increased number of dots could be seen by PLA using anti-PA28γ and coilin antibodies, supporting the notion that PIP30 inhibits PA28γ/coilin interaction (SI Appendix, Fig. S8).

Altogether, these experiments show that PIP30 effect on CBs is mediated through its ability to bind PA28γ and to counteract PA28γ association with coilin, confirming that PIP30 indeed functions as an endogenous regulator of PA28γ.

Discussion

Despite the fact that PA28 complexes have long been known, little is understood regarding their roles as proteasome regulators, besides their ability to open upon binding the gated channel of the 20S proteasome. However, because specific protein substrates have been described for PA28γ, it is possible that these regulators are not just passively opening the gate of the 20S proteasome but somehow contribute to substrate selection and possibly unfolding and injection into the 20S core. If true, then it is likely that they work in synergy with other proteins, because by themselves they seem a priori unable to actively promote proteasomal...
Saccharomyces cerevisiae Coi1n IP has 222 interaction. Pull-downs were performed with extracts of proteasome inhibitor-treated cells (Dataset S5), confirming both that (i) PA28γ/20S proteasome interaction is labile (31) and is stabilized upon proteasome inhibition (11, 43), and (ii) PA28γ, as this has been described for PA28γβ, can also be recruited in hybrid forms of the proteasome in which the 20S proteasome is bound to the 19S complex on one side and PA28γ on the other side.

Importantly, we identified a prominent partner of PA28γ, PIP30/FAM192A. Interaction between PIP30 and PA28γ has already been listed in large-scale proteomics experiment data in Drosophila and human (44, 45). However, our results validate its biological relevance.

We demonstrated that this interaction is direct and occurs in cells with 20S proteasome-free and -bound PA28γ. The interaction is specific for PA28γ because we found no interaction between PIP30 and PA28γβ in vitro. This suggests that the interaction may involve the homolog-specific insert, which is the most divergent domain between PA28 paralogs (46). Importantly we show that the PA28γ-binding region of PIP30 is located in its C-terminal end and that CK2 phosphorylation of a short motif in this region stabilizes its association with PA28γ. Because mutation of serine residues (S219 and S222) of both typical CK2 targets does not completely abolish binding, it is likely that additional serine residues are phosphorylated by CK2. This is in line with the ability of CK2 to catalyze the generation of phosphoserine stretches (36), and in agreement with our proteomics analyses (SI Appendix, Fig. S5) and with the report that S219, S221, S222, and S224 of PIP30 are phosphorylated in vivo (Global Proteome Machine, Accession: ENSP00000335808 [47]). The role of these multiple phosphorylation events is not clear. CK2 is indeed a ubiquitous and pleiotropic kinase (48), and even if its activity has been shown to be positively regulated by the Wnt/β-catenin pathway (49) it is considered to be constitutively active in cells. Our results also suggest an extremely slow turnover of the phosphate groups for the majority of PIP30 bound to PA28γ, probably because steric hindrance makes them inaccessible to phosphatases once the complex is formed. It thus seems unlikely that CK2 phosphorylation of PIP30 is used in cells to dynamically control PIP30/PA28γ interaction.

Comparison of PIP30 and PA28γ distributions within the eukaryotic phylogenetic tree (SI Appendix, Fig. S3B) shows that their presence or absence is not correlated. For example, in all Ascomycota, including Saccharomyces cerevisiae, PIP30 is present while PA28γ has been lost. This indicates that the two proteins may function independently of each other. However, in human cells, more than 70% of PA28γ and PIP30/FAM192A are associated in the same complex (SI Appendix, Fig. S1B), suggesting that they cooperate in most of their functions.

In line with this assertion, our data show that PIP30 regulates the function of PA28γ as a proteasome regulator, since its inactivation halves the intracellular level of the PA28γ/20S proteasome complex (Fig. 1 D and E). However, because the 20S proteasome/PA28γ complex only represents ≤5% of the total 20S proteasome (29), it is difficult to predict the biological impact of this effect. Based on our in vitro analyses showing that PIP30 inhibits the degradation of some peptides by reconstituted 20S proteasome/PA28γ complexes but is permissive for others (Fig. 3), it is tempting to speculate that PIP30 acts as a molecular sieve that hinders entrance of some protein substrates into the PA28γ channel while being neutral for others. The likely binding of PIP30 to the PA28γ-specific insert that forms a loop located close to the pore of the complex (25, 50) is compatible with such a role. Confirmation and further dissection of this hypothesis requires comparison of proteasome- and PA28γ-dependent substrates whose degradation is differentially regulated by PIP30. Unfortunately, among the several described PA28γ substrates that we tested, only p21 degradation proved to be PA28γ-dependent in our conditions, and the cellular stability of this protein turned out to be insensitive to PIP30 presence or absence.

Interestingly, the notion that PA28 complexes could act as a molecular sieve has already been put forward for PA28γβ, based on detailed studies showing that it selectively filters peptide substrates. We thus performed various proteomics experiments to characterize proteins associated with PA28γ.

Among the interaction partners of endogenous PA28γ, we found several proteins involved in the regulation of RNA processing (Dataset S1), suggesting an important role of PA28γ in this function. Although PA28γ interacts with the 20S proteasome, we identified only the α6 subunit (PSMA1) in the conditions used. However, we found most 20S proteasome as well as 19S complex subunits with high H/L ratios when PA28γ pull-downs were performed with extracts of proteasome inhibitor-treated cells (Dataset S5), confirming both that (i) PA28γ/20S proteasome interaction is labile (31) and is stabilized upon proteasome inhibition (11, 43), and (ii) PA28γ, as this has been described for PA28γβ, can also be
products coming out of the proteasome (8, 51). This raises the possibility that PIP30 could, together with PA28γ, play a selective role not only on substrate entrance but also peptide release by the proteasome.

Taken together, our data are compatible with the interesting hypothesis that binding of PIP30 to PA28γ could induce a global conformational change of the latter that would both favor its binding to the 20S proteasome and alter its substrate selectivity through alteration of the entrance or the structure of its channel. Structure/functions analyses aiming at precisely mapping the consequences on PA28γ of PIP30 binding as well as detailed characterization of peptides produced by the proteasome in the presence of PA28γ and PA28γ/PIP30 complexes will help to answer these questions.

A second illustration of the role of PA28γ as a PA28γ regulator is the demonstration that both proteins intimately cooperate in the regulation of CB integrity. Indeed, PAIP30 depletion leads to a strong decrease in the number of CBs and to an increased interaction between coilin and PA28γ (Fig. 5B and SI Appendix, Fig. S8 A and B). This mimics the phenotype induced by PA28γ overexpression (21) (Dataset S2). PAIP30 depletion also elicits the accumulation of PA28γ in residual CBs, while PA28γ is usually not detectable in these structures. To our knowledge, PA28γ has only been observed in CBs of motor neurons from patients with type I spinal muscular atrophy (52). In these pathologic neurons, the assembly of CBs is impaired, due to the lack of the survival motor neuron protein, an essential CB component (53–55). Because CBs are dynamic structures undergoing constant remodeling (56), the accumulation of PA28γ in residual CBs observed in both PA28γ−/− cells and spinal muscular atrophy motor neurons could reflect the fact that these residual CBs are stalled at transient intermediate stages of assembly/disassembly in which PA28γ is involved. Alternatively, the absence of PAIP30 may result in the formation of defective CB structures that are not normally present in wild-type cells. Interestingly, the proteasome is not recruited into CBs, together with PA28γ, in the absence of PAIP30 (SI Appendix, Fig. S9A), and proteasome inhibition—which greatly enhances the binding of PA28γ to the 20S core (11)—inhibits the accumulation of PA28γ into CBs (SI Appendix, Fig. S9B). These observations raise the possibility that the functions of PA28γ and PAIP30 in CBs could be proteasome-independent.

Taking these data together, the effects of PAIP30 deletion on CB integrity suggest that increasing the levels of PA28γ/coillin complexes negatively regulates the number of CBs and that PAIP30 inhibits the association between PA28γ and coilin. This model supports the idea that PA28γ overexpression leads to CB destabilization by overwhelming PAIP30 inhibition, and therefore that the equilibrium between free PA28γ and PAIP30-bound PA28γ is an important parameter in this process. In this regard, it is interesting to note that CB fragmentation upon UV-C treatment is associated with the concomitant increase in PA28γ and its recruitment to coilin (21). If PAIP30 levels are limiting in cells, the resulting excess of PAIP30-free PA28γ might be sufficient to interact with coilin and induce CB fragmentation. However, it is difficult to imagine that PA28γ functions in CBs could be taken in charge only by neosynthesized PA28γ that would have escaped definitive PAIP30 inhibition, it seems more likely that the binding of PAIP30 to PA28γ can be negatively regulated to favor their dissociation when needed. As mentioned above, our data do not support the idea that such regulation could be mediated only by modulation of CK2 activity or by dephosphorylation of CK2 sites on PAIP30. Therefore, we speculate that other regulatory mechanisms have yet to be discovered, must be at play to dissociate the PA28γ/PIP30 complex. Because the interaction zone between PAIP30 and PA28γ is protected against phosphatases, destabilization of the interaction is likely to occur through conformational changes incompatible with further binding. In principle, such conformational changes could be mediated by posttranslational modifications of either PA28γ or PI P30, or by binding of additional partners or chaperones able to alter upon binding the structure of the complex. Additional work is required to clarify this issue.

Taken together, the results presented here show that PAIP30 is an important partner of PA28γ that regulates its interactome and therefore its functions, for example, by stabilizing its interaction with the 20S proteasome and inhibiting its interaction with coilin. Although most of the data presented in this report show an inhibitory role of PAIP30 toward PA28γ functions, the fact that PAIP30 does not influence the PA28γ-dependent degradation of the protein p21 shows that PAIP30 is not a general inhibitor of PA28γ. In any case, because most PA28γ is bound to PI P30 in standard cell culture conditions, it is clear that PAIP30 must now be taken into account when studying PA28γ. Although PAIP30 functions are yet to be understood regarding PAIP30 biological functions, our results represent a significant breakthrough because they provide clues on the regulation of PA28γ/20S proteasome complex, as well as angles of attack to dissect PA28γ functions and mechanisms of action.

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