Identification of Nuclear Phosphatidylinositol 4,5-Bisphosphate-Interacting Proteins by Neomycin Extraction*§

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Considerable insight into phosphoinositide-regulated cytoplasmic functions has been gained by identifying phosphoinositide-effector proteins. Phosphoinositide-regulated nuclear functions however are fewer and less clear. To address this, we established a proteomic method based on neomycin extraction of intact nuclei to enrich for nuclear phosphoinositide-effector proteins. We identified 168 proteins harboring phosphoinositide-binding domains. Although the vast majority of these contained lysine/arginine-rich patches with the following motif, K/R-(Xn = 3–7)-K-X-K/R-K/R, we also identified a smaller subset of known phosphoinositide-binding proteins containing pleckstrin homology or plant homeodomain modules. Proteins with no prior history of phosphoinositide interaction were identified, some of which have functional roles in RNA splicing and processing and chromatin assembly. The remaining proteins represent potentially other novel nuclear phosphoinositide-effector proteins and as such strengthen our appreciation of phosphoinositide-regulated nuclear functions. DNA topology was exemplar among these: Biochemical assays validated our proteomic data supporting a direct interaction between phosphatidylinositol 4,5-bisphosphate and DNA Topoisomerase IIα. In addition, a subset of neomycin extracted proteins were further validated as phosphatidylinositol 4,5-bisphosphate-interacting proteins by quantitative lipid pull downs. In summary, data sets such as this serve as a resource for a global view of phosphoinositide-regulated nuclear functions. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.003376, 1–15, 2011.

Phosphoinositides (PIs)¹ are lipid second messengers unique among phospholipids: Their inositol head group is rapidly phosphorylated by specific lipid kinases yielding seven distinct biologically relevant phosphatidylinositol derivatives. The coordinated activities of the PI-specific kinases and phosphatases generate PI profiles, which contribute to downstream signaling events regulating a variety of cellular processes such as proliferation, cell survival, migration, and vesicular trafficking (1–4). Impairment of PI metabolism is associated with cancer as well as neurological and immunological disorders (5–7). PIs are not only substrates for the generation of second messengers but are also second messengers themselves. They have emerged as sensors for specific PI-binding domains present in a diverse array of proteins: PH (pleckstrin homology), epsin N-terminal homology, FYVE (Fab1, YOTB, Vac1, EEA1), Phox homology, PHD (plant homeodomain), PDZ domains as well as unstructured lysine/arginine-rich patches. These domains display a range of heterogeneity in terms of their specificity for the different PIs (8–10) and recruit target, domain-containing, effector proteins in a temporal and spatial manner to sites of PI synthesis at many cytoplasmic locations (11).

PIs (notably phosphatidylinositol(3)phosphate (PtdIns(3)P), PtdIns(4)P, PtdIns(5)P, PtdIns(4,5)P₂, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃) have also been identified within the confines of the nucleus, together with the enzymes responsible for their metabolism (12–18). They are regulated independently of the cytoplasmic PI pool (19, 20) and have been localized to defined nuclear regions (21–25). Emerging data point to significant roles for nuclear PIs. Increases in nuclear PtdIns(5)P mass levels result in the nuclear localization of the transcrip-

¹ The abbreviations used are: PI, phosphoinositides; NLS, nuclear localization sequence; PH, pleckstrin homology; PHD, plant homeodomain; PLC, phospholipase C; PtdIns(3)P, phosphatidylinositol 3-monophosphate; PtdIns(4)P, phosphatidylinositol 4-monophosphate; PtdIns(5)P, phosphatidylinositol 5-monophosphate; PtdIns(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)P₃, phosphatidylinositol 3,5-bisphosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; SILAC, stable isotope labelling with amino acids in cell culture; SPR, surface plasmon resonance; Topo IIα, DNA Topoisomerase IIα.
tion factor inhibitor of growth protein 2 via an interaction between its PHD domain and the lipid (26, 27). Nuclear PtdIns(4,5)P$_2$ binds to and regulates the activity of the poly(A) polymerase Star-PAP (nuclear speckle targeted PIPKIIα regulated-poly(A) polymerase), an enzyme that also binds directly to the enzyme responsible for the synthesis of PtdIns(4,5)P$_2$, namely, the Type I PtdIns(4)P 5-kinase (28). The chromatin remodeling protein BRG1 binds to PtdIns(4,5)P$_2$ (29, 30), whereas other data link nuclear PIs to cell cycle progression (31, 32), apoptosis via an interaction between nucleophosmin and PtdIns(3,4,5)P$_3$ (33), and pre-mRNA processing via interaction of nuclear speckle pools of PtdIns(4,5)P$_2$ (22–25). Nuclear speckles are enriched in small nuclear ribonucleoproteins (snRNPs) and splicing factors (34) and constituent proteins that have been identified as PtdIns(4,5)P$_2$ effector proteins, such as Syntenin-2 (35). These authors suggest that this interaction sequesters PtdIns(4,5)P$_2$ to these nuclear structures. Furthermore the mRNA export factor Aly binds to both PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$, an interaction essential for its localization to nuclear speckles (36).

Together these data point to a diverse set of nuclear activities regulated, in part, by the presence of PIs within this organelle. The global significance of nuclear PI-protein interactions however is still poorly understood, due largely to few known nuclear effector proteins. However in cases where this has been investigated in detail, it appears that such interactions have profound physiological effects (27–29). The identification of other nuclear PI effectors is therefore likely to shed more light on these and importantly other nuclear PI functions. To address this we have developed a proteomic approach to enrich for and identify potential nuclear PI-binding proteins by nano liquid chromatography (LC)-tandem MS (MS/MS). We have used the aminoglycoside neomycin to pull out this subset of proteins from isolated, intact nuclei and combined this approach with quantitative MS to determine specificity of the extraction procedure. Lipid affinity matrices were used to validate, biochemically, our proteomics data on target proteins and bioinformatics clustering analysis of the identified proteins allowed us to speculate the functional significance of nuclear PIs on a proteomic scale. Using this principle, we have identified 349 nuclear proteins, 48% of which harbor PI binding domains. Clustering analysis of these proteins has been investigated in detail, it appears that such interactions have profound physiological effects (27–29). The identification of other nuclear PI effectors is therefore likely to shed more light on these and importantly other nuclear PI functions. To address this we have developed a proteomic approach to enrich for and identify potential nuclear PI-binding proteins by nano liquid chromatography (LC)-tandem MS (MS/MS). We have used the aminoglycoside neomycin to pull out this subset of proteins from isolated, intact nuclei and combined this approach with quantitative MS to determine specificity of the extraction procedure. Lipid affinity matrices were used to validate, biochemically, our proteomics data on target proteins and bioinformatics clustering analysis of the identified proteins allowed us to speculate the functional significance of nuclear PIs on a proteomic scale. Using this principle, we have identified 349 nuclear proteins, 48% of which harbor PI binding domains. Clustering analysis of these proteins revealed overrepresented functions related to RNA splicing, chromatin assembly, and DNA topological change. Furthermore, we have validated this method first by identifying a subset of proteins displaced by neomycin as PtdIns(4,5)P$_2$ interacting proteins using quantitative lipid pull downs and second by biochemically characterizing the interaction of PtdIns(4,5)P$_2$ with DNA topoisomerase IIα (Topo IIα), an enzyme with hitherto indirect links with nuclear PI metabolism. This proteomics approach provides compelling evidence suggesting that nuclear PIs interact with a wide range of nuclear proteins regulating numerous nuclear functions.

**EXPERIMENTAL PROCEDURES**

**Reagents**

**Lipid preparation**—Phosphoinositide lipids used for surface plasmon resonance (SPR) analysis were purchased from Cell Signals Inc. L-α-ω-my-o-phosphatidylinositol (PtdIns), L-α-ω-my-o-phosphatidylinositol 3-monophosphate (PtdIns3P), L-α-ω-my-o-phosphatidylinositol 5-monophosphate (PtdIns5P), L-α-ω-my-o-phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P$_2$), and L-α-ω-my-o-phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) were stored as dry aliquots of 0.5 nmol at −70 °C. For SPR analysis, lipid aliquots were equilibrated to room temperature, before being rehydrated in 100 μl 10 mM Tris pH 7.4 for 1 h. The solution was sonicated at high frequency for 5 min (cycles of 15 s ON and 7.5 s OFF) using a Bioruptor UCD200 (Diagenode, Denville, NJ). Finally, 200 μl 50 mM HCl were added and lipids were loaded onto an activated gas-liquid chromatography sensor chip at 30 μl/min for 300 s. Phosphoinositide lipids used in competition in either lipid pull downs or DNA decatenation assays were short chain PIs of eight carbons from Echelon Biosciences Inc. (Salt Lake City, UT).

**Protein Purification**

**Expression and Purification of GST-Tagged Proteins Used in Lipid Pull Downs**—Purification of GST-PLCδ1, PH and GST-GRP1-PH was as described (39) but with some modifications. GST-PLCδ1-PH and GST-GRP1-PH constructs were transformed into Escherichia coli strain BL21 RIL DE3 and bacterial cultures were grown at 37 °C and induced with 0.5 mM isopropyl-$β$-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Bacterial pellets were resuspended in 50 mM Tris pH 7.5, 1 mM EDTA, 0.27 mM sucrose, 1% Triton X-100, 1 mM Na$_3$VO$_4$, and 2 mM NaN$_3$, including 1 mM dithiothreitol and complete protease inhibitor mixture from Roche added fresh, lysed by one round of freeze-thawing and sonication and debris were removed by centrifugation. GST-tagged proteins were purified with glutathione-agarose 4B beads from an overnight pull down, and analyzed by SDS-PAGE and Coomassie staining for purity.

**Expression and Purification of GST-Tagged Proteins Used in SPR Analyses**—A glycerol stock of E. coli DH5α cells transformed with the corresponding GST-protein construct was used to inoculate 10 ml LB supplemented with 100 μg/ml ampicillin. The culture was grown overnight at 37 °C with shaking. Cells were diluted 10-fold and grown for another 2 h at 37 °C and induced with 100 μM isopropyl-$β$-D-thiogalactopyranoside at 25 °C overnight. Cells were collected by centrifugation at 4000 rpm for 15 min and lysed into 2 ml ice-cold lysis buffer (PBS, 1% Triton X-100, and 10 mM dithiothreitol). The lysate was centrifuged in liquid nitrogen, thawed at 37 °C and sonicated using a
Bioruptor UCD200 (Diagenode). The lysate was cleared by centrifugation at 4 °C and the supernatant was mixed with 50 μl packed glutathione-Sepharose 4B beads (GE Healthcare), previously equilibrated in lysis buffer. The mixture was rotated at 4 °C for 1.5 h, the beads were collected by centrifugation and washed three times with lysis buffer followed by two washes with wash buffer (50 mM Tris pH 8.0 and 300 mM NaCl). Proteins were eluted with 240 μl elution buffer (wash buffer supplemented with 10 mM reduced glutathione and 10 mM dithiothreitol). Aliquots were snap frozen in liquid nitrogen and kept at −70 °C.

**Purification of His-Topo IIα**—COS-1 cells were transfected with EGFP-RGS-6xHis-Topoll plasmid and lysed with buffer A (50 mM NaH2PO4 pH 8, 300 mM NaCl, 0.5% Tween 20, including 20 mM imidazole and protease inhibitors) with one freeze-thaw cycle. Cell extracts were incubated with Ni-NTA agarose beads for 16 h at 4 °C on a rotator. Sedimented beads were washed five times with buffer B (same as buffer A but with 0.1% Tween 20) and Topo IIα was eluted with buffer B containing 250 mM imidazole. Topo IIα was identified by MS and detected by Western immunoblotting.

**Cell Preparation**

**Cell Culture and SILAC (Stable Isotope Labeling with Amino Acids in Cell Culture) Labeling**—Jurkat cells were grown in RPMI 1640 and 10% FCS and MEL cells in Dulbecco modified Eagle medium (DMEM) and 10% FCS in 5% CO2 at 37 °C. For isotopic metabolic labeling, MEL cells were transferred to DMEM (minus lysine and arginine) supplemented with 10% dialyzed fetal bovine serum with either 13C L-arginine and 15N L-lysine (light medium) or 13C L-arginine and 13C L-lysine (heavy medium) and subcultured for at least 10 population doublings that ensured complete incorporation of isotopic amino acids.

**Nuclear Fractionation and Neomycin Extraction**—Intact nuclei were prepared as described previously (32, 40) in the absence of Triton X-100. An equivalent number of nuclei (10^6 nuclei per 100 μl) were washed twice with retention buffer (20 mM Tris pH 7.5, 70 mM NaCl, 20 mM KCl, 5 mM MgCl2, and 3 mM CaCl2) (41) and incubated in the same buffer in the absence (control) or presence of 5 mM neomycin (trisulfate salt, Sigma) for 30 min at room temperature. Nuclei were sedimented by centrifugation at 13,000 rpm for 5 min and supernatants were collected and used in Western analyses or in quantitative proteomic analyses. For quantitative proteomic analyses (Fig. 2), nuclei obtained from 12C-labeled cells were incubated with neomycin whereas nuclei obtained from 13C-labeled cells were incubated with retention buffer only (2.5 × 10^5 cells were used per condition).

Western immunoblotting: Proteins were resolved on 4%–12% NuPAGE Novex Bis-Tris gels with MOPS running buffer (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk, incubated with primary antibodies overnight at 4 °C and with secondary antibodies conjugated to HRP for 1 h at room temperature. Revelation was performed by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and scanned with a LAS-3000 imaging system (Fuji).

Multi fractionation of proteins: Nuclear supernatants were dried down by vacuum centrifugation and resuspended in 200 μl of 6 M urea and 1% acetic acid. Proteins were separated by high performance liquid chromatography (HPLC) on a macroporous C18 reverse phase column (Agilent Technologies) with a 30 min multistep gradient elution of buffer A (2% acetonitrile, 0.1% trifluoroacetic acid) to buffer B (95% acetonitrile, 0.08% trifluoroacetic acid) at 300 μl/min. Eight fractions were collected, dried down, and resuspended in SDS sample buffer before separation by SDS-PAGE.
Protein-PI Interaction Analyses—Lipid pull downs: Purified-recombinant GST-tagged proteins (2 µg for GST-PLCδ1-PH and GST-GRP1-PH) or EGFP-His-Topo IIa full length (5 µg) were diluted in 400 µl of binding buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 5 mM β-glycerophosphate, 1 mM orthovanadate and 2 mM NaF) with 20 µl PI-conjugated agarose beads or control agarose beads (Echelon Biosciences Inc) for 1 h at 4 °C. Sedimented beads were washed twice with the same buffer and proteins were eluted and resolved by SDS-PAGE. For competition studies with free lipids, short chain C8 PtdIns(4,5)P2 was incubated with recombinant proteins for 15 min on ice prior to the addition of the lipid beads. For lipid pull down assays of control and neomycin supernatants, three rounds of dialysis in binding buffer using Slide-A-Lyser® Mini dialysis units (Pierce) was carried out. For the quantitative lipid pull downs, dialyzed neomycin extracts (750 µg) obtained from either 13C-labeled cells or 15C-labeled cells were incubated with control-conjugated beads and PtdIns(4,5)P2-conjugated beads respectively.

Surface Plasmon Resonance (SPR)—A ProteOn XPR36 (BioRad) was used for SPR analysis. To prepare hydrophobic SPR sensor chips, undecylamine (C11H23N) was covalently coupled to a GLC sensor chip (BioRad) using a two-step amine coupling procedure in PBS running buffer at 25 °C. After cleaning the sensor surface with two horizontal and two vertical injections of 0.5% 3-[3-cholamido-propyl]dimethylammonio]-1-propanesulfonic acid (CHAPS) at 100 µl/min for 36 s, a freshly mixed solution of 50 mM sulfo-NHS and 200 mM EDC was injected at 30 µl/min for 360 s. Undecylamine (Sigma Aldrich) was prepared for immobilization by making up a 1% solution in dimethyl sulfoxide, which was then diluted 20-fold in 10 mM sodium acetate pH 5.0 and injected at 30 µl/min for 400 s in the second step of the immobilization procedure. One molar ethanolamine hydrochloride pH 8.5 injected at 30 µl/min for 360 s was used to deactivate excess activated carboxylic groups and to remove electrostatically bound undecylamine.

After the sensor surface was preconditioned with two injections of 50 mM NaOH at 100 µl/min for 36 s, phosphoinositolipid were injected onto the activated GLC sensor chip surface at 30 µl/min for 300 s at 25 °C. If necessary this step was repeated until the desired lipid loading level was obtained. The ligand sensor surface was post-conditioned with one injection of 1 nM NaCl and two injections of 50 mM NaOH at 100 µl/min for 18 s.

n=2 s on each precursor ion or stopped when the signal intensity fell below ten counts per second respectively before a new MS to MS/MS cycle was started. Fused silica capillaries with 20 µm inner diameter served as spray emitters. Precursors were excluded from MS/MS experiments for 1 min and singly charged ions were excluded as precursors for MS/MS. Raw data were processed using MassLynx 4.0 ProteinLynx and the MS/MS data exported in Micromass pik format.

The LTO-Orbitrap XL was coupled to a Ultimate 3000 nano-HPLC system (Dionex, Sunnyvale, CA) fitted with an Acclaim PepMap 100 column (C18, 3 µm, 100 Å) (Dionex, Sunnyvale, CA) with a capillary of 12 cm bed length. A flow rate of 300 nl/min was employed with a solvent gradient of 7% B to 35% B in 77 min and subsequently to 50% B in 10 min. Solvent A was 0.1% formic acid, whereas aqueous 90% acetonitrile in 0.1% formic acid was used as solvent B. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with resolution r = 60,000 at m/z 400 (after accumulation to a target of 10,000,000 charges in the LTQ). The method used allowed sequential isolation of the most intense ions, up to six, depending on signal intensity, for fragmentation on the linear ion trap using collision-induced dissociation at a target value of 100,000. For accurate mass measurements the lock mass option was enabled in MS mode and the polydimethylcyclosiloxane ions generated in the electrospray process from ambient air were used for internal recalibration during the analysis. Target ions already selected for MS/MS were dynamically excluded for 60 s. General mass spectrometry conditions were: electrospray voltage, 1.5 kV; no sheath and auxiliary gas flow. Ion selection threshold was 500 counts.
for MS/MS, and an activation Q-value of 0.25 and activation time of 30 ms were also applied for MS/MS.

Raw files were processed to generate mascot generic files using the program DTASupercharge v.1.29 and merged with MGB combiner to obtain one mgf file per gel lane.

Computational Analyses

Database Search—The resulting pkl files (International Protein Index) (Q-ToF) and mgf files (LTQ-orbitrap) were searched against the IPI mouse database v3.46 (55272 entries) using the Mascot search engine version 2.1.04. Possible structure modifications allowed were carbamidomethyl cysteine as a fixed modification. Oxidized methionine, deamidation of Asn and Gln, 13C(6) Lys and Arg were searched as variable modifications. Searches were done with tryptic specificity allowing up to 1 miscleavage and a tolerance on mass measurement of 75 ppm in MS mode and 0.4 Da for MS/MS ions (Q-ToF), and 7 ppm in MS mode and 0.4 Da for MS/MS ions (LTQ-orbitrap). Only peptide identifications with a p value < 0.05 and with ion scores over 42 (as determined by Mascot), and protein identifications based on no less than two peptides were used further. The false discovery rate was 0.082% using a reversed database under the same conditions.

Protein Quantitation—Protein quantitation was achieved using the MSQuant software v1.4.2a23 (http://msquant.sourceforge.net/), using raw data files together with the corresponding HTML result files generated with Mascot. Peptide ratios were calculated for each arginine- and/or lysine-containing peptide as the peak intensity of 13C labeled arginine/lysine divided by the peak intensity of nonlabeled 12C arginine/lysine (13C/12C) for each single-scan mass spectrum. Peptide ratios obtained for each protein were averaged and the standard deviation determined. Only proteins identified with LOG2 ratios < -0.5 (Fig. 2) and LOG2 ratios > 0.5 (Fig. 4) were kept.

The results were exported to Excel for further analysis. Statistical analyses were performed with StatQuant (42) for the quantitative lipid pull down.

Bioinformatic Analyses—Searches for K/R motifs: the IPI accession numbers were extracted and submitted to the Sequence Retrieval System (SRS) (http://srs.ebi.ac.uk/) and the corresponding fasta format sequences were retrieved. The fasta formatted sequences file was then parsed by an in-house script (using Python 2.5.2 (3)) to find occurrences of the following pattern K/R-X(n3–7)-K-X-K/R-K/R.

For the biological networks analyses, the identified IPI entries were first reassigned to gene entries (EntrezGene ID) annotated in the EnsEMBL database using Biomart (www.ensembl.org) (43). Gene Ontology (GO) identifiers were assigned to each IPI/gene entry and overrepresentation of GO categories were statistically analyzed using Cytoscape 2.6.1 and its plug-in BiNGO 2.0 (Biological Networks Gene Ontology tool) (44) by comparing the GO annotations obtained in our dataset to those of the mouse genome restricted to entries annotated to the nucleus compartment (GO :0005634). The representation for each GO category was calculated as the ratio between the cluster (neomycin dataset) frequency and the reference dataset (murine nucleome) frequency, the frequency being the percentage of gene entries in a particular GO term category compared with the respective total number of entries. These analyses were performed for both biological processes and Molecular functions. The significance of overrepresented GO categories was assessed with a hypergeometric test and the Benjamini and Hochberg false discovery rate correction. A corrected p value < 0.05 was considered significant and only significantly overrepresented GO categories are presented (Fig. 3).

The functional classification of PtdIns(4,5)P2 interacting proteins and the over-representation of their biological processes presented in Fig. 2 were performed using PANTHER classification system/gene expression analysis (http://www.pantherdb.org/tools/genexAnalysis.jsp) (45, 46).

RESULTS

Extraction of Potential Nuclear PI-Binding Proteins with Neomycin—Neomycin (Fig. 1A) is known to bind to PIs with high affinity by way of electrostatic interactions between the basic amino groups of the compound and the negatively charged phosphate groups of PIs (47–50). Proteins that bind to PIs do so via similar interactions involving basic residues found in their PI-binding domains (9, 51). We speculated therefore that neomycin should compete for PI binding with PI-binding proteins, displacing them from their site of interaction. To establish if neomycin can displace PI bound proteins, immobilized PtdIns(4,5)P2 affinity beads were incubated with GST-tagged PH domain of PLC δ1 (Fig. 1B). In the absence of any competing lipids GST-PLCδ1-PH bound efficiently to PtdIns(4,5)P2 affinity beads. However, in the presence of free PtdIns(4,5)P2 and the water soluble head group, Ins(1,4,5)P3, this interaction was completely abolished. PtdIns(3,4)P2 was unable to compete for binding whereas only partial binding was observed in the presence of PtdIns(3,5)P2, demonstrating specificity for PtdIns(4,5)P2. Importantly, in the presence of neomycin the binding of GST-PLCδ1-PH domain was significantly reduced. These data demonstrate that neomycin can compete with characterized PI binding domains for binding to their target lipids. We extended these findings to isolated intact nuclei preparations. Supernatants recovered from nuclei incubated in the absence or presence of neomycin showed marked differences in their protein composition, as assessed by Coomassie blue staining (Fig. 1C). Protein bands were visible in control (no neomycin) supernatants, presumably as a result of nonspecific extraction or leakage. In contrast many more protein bands were visible from supernatants obtained following neomycin incubation. These differences in protein extraction were the same even when nuclei were prepared in the presence of 0.1% Triton X-100 (Supplemental Fig. S1), suggesting that the majority of these proteins are not extractable by detergent and derive from within the nuclear matrix. MS was used to identify prominent proteins specific for the neomycin extract (Topo IIα, NF45, nucleophosmin and histone H1; Fig. 1C), which were validated by Western immunoblot analyses (Fig. 1D). In contrast nonspecific proteins, such as the heterogeneous nuclear ribonucleoprotein (hnRNP) DOB, were equally present in supernatants obtained from nuclei incubation with or without neomycin.

Identification of Neomycin-Displaced Nuclear Proteins—To establish a substantial inventory of proteins that are specifically displaced from intact nuclei by neomycin, we have combined a SILAC-based quantitative proteomic approach with our extraction procedure. The workflow is outlined in Fig. 2A. An equal number of isolated nuclei from 12C and 13C labeled cells were extracted with neomycin and buffer respectively. The supernatants were combined at equal protein concentration and the protein complexity of the sample reduced by an
initial round of fractionation using macroporous C18 reverse phase LC. Resulting fractions were further separated by one-dimensional SDS-PAGE, sliced and digested with trypsin. Peptides were analyzed by LC-MS/MS and isotope ratios for each peptide were quantified using MSQuant. See Table S1 for the identified proteins.

B. Peptides identified and quantified for Topo IIα listing their respective Mascot score and 13C/12C ratio. C. Example for the first peptide (ELILFSNSDNER), doubly charged, which is about 40 times more abundant in the light form than in the heavy form. D. Protein classification according to their Log2 13C/12C ratios: proteins with Log2 > 0.5 (displaced by buffer, 64 proteins), Log2 < −0.5 (displaced by neomycin, 349 proteins) and proteins with −0.5 < Log2 < 0.5 (equally displaced by buffer and neomycin, 30 proteins).

proteins were found to harbor PHD or PH domains (dynamin-2, Bruton’s tyrosine kinase (BTK), PHD finger protein 8 (Ph8), the E3 ubiquitin ligase UHRF1, Fbx11 (or 133 kDa protein), Src kinase associated phosphoprotein 2 (Skap2) and Rho/Rac guanine exchange factor 2 (ArhGEF2)), whereas 165 proteins, contained at least one K/R-rich motif (K/R-(Xₙ=3–7)-K-X-K/R-K/R), known to bind PtdIns(4,5)P₂ in several effector proteins (52) (Supplemental Table S1). Overall, 48% of all proteins displaced by neomycin displayed a K/R motif. In comparison, 18.7% of the proteins displaced by buffer alone contained such motifs (Fig. 2D). In addition, 30.2% of all known proteins that are annotated to the nucleus contain K/R motifs. The use of neomycin achieved therefore a 1.6-fold enrichment of proteins containing a K/R motif.

Functional Characterization of Neomycin-Displaced Proteins—To have some functional insight of our data, each of the 349 neomycin specific proteins was annotated by a Gene Ontology (GO) database for biological processes and molecular functions. The over-representation of each GO category was determined using Cytoscape and its Plug-in BiNGO, in the context of GO categories annotated to the nuclear com-
Neomycin Extracts—Our previous data suggests that within the neomycin extracted proteins exits a subset of proteins that bind directly to PI, in particular PtdIns(4,5)P2. Quantitative lipid pull-down experiments were run to identify specific PtdIns(4,5)P2-interacting proteins from neomycin supernatants (Fig. 4A). Neomycin supernatants obtained from either 12C and 13C-labeled cells were incubated with control beads (beads conjugated to the fatty acid moieties only) or PtdIns(4,5)P2-conjugated beads respectively. Quantitative and statistical analyses revealed the identification of a total of 70 proteins, of which 28 proteins, annotated to the nucleus compartment, were specifically pulled down by PtdIns(4,5)P2 beads. These proteins are listed in Table I. Nineteen of these harbor a K/R motif that represented a twofold enrichment of PtdIns(4,5)P2 interacting proteins containing such motifs compared with noninteracting proteins (Fig. 4B). Functional classification of these proteins pointed to roles in mRNA transcription regulation, mRNA splicing and protein folding (Fig. 4C), the last two categories being over-represented (Fig. 2D) in this dataset.

To demonstrate an interaction between Topo IIα and PIs, neomycin supernatants were dialyzed (removing neomycin) and incubated with control beads or beads conjugated with either PtdIns(4,5)P2 or PtdIns(3,4,5)P3 (Fig. 5A). Proteins bound to lipid beads were eluted and resolved by SDS-PAGE. A positive band for Topo IIα by Western blotting showed the interaction with PtdIns(4,5)P2-conjugated beads, whereas binding to PtdIns(3,4,5)P3-conjugated beads was marginally greater than control beads (Fig 5A). These data demonstrated specificity for binding to PtdIns(4,5)P2. As a control, specific interactions with PtdIns(4,5)P2 or PtdIns(3,4,5)P3 beads were demonstrated by lipid pull-down assays of known proteins harboring PH domains interacting specifically with either PtdIns(4,5)P2 or PtdIns(3,4,5)P3, i.e. PLCδ-PH and GRP1-PH respectively (Fig 5B). The same analysis was performed with purified Topo IIα. In this case, Topo IIα specifically bound to PtdIns(4,5)P2 whereas binding to PtdIns(3,4,5)P3 was at control level (Fig 5C). We also investigated the ability of free PIs to compete with PtdIns(4,5)P2-beads for binding to purified

department. Categories that were significantly over-represented statistically are shown in Figs. 3A and 3B (p < 0.05). These categories are also visualized as interaction networks and appear in downstream nodes in Supplemental Figs. 2 and 3. The GO categories, DNA topological change (represented by DNA topoisomerases) and nucleosome positioning (represented by histones) are particularly over-represented in the neomycin dataset (Fig. 3A). Several categories in molecular functions were also over-represented (Fig. 3B) and may represent functions attributed to PI effector proteins. We performed the same analyses, but on the subset of proteins containing PI-binding motifs, which resulted in the discovery of several common categories with the whole list (shown as gray bars in Figs. 3A and 3B and also visualized as blue circles in GO hierarchy networks in Supplemental Figs. 2 and 3). The proteins present in these categories are listed in Supplemental Tables S2 and S3. mRNA processing and especially RNA splicing, as well as chromatin assembly, were found to be significantly overrepresented, consistent with other data (24, 28, 53). The GO category, DNA topological change, was highlighted in our analysis and to our knowledge proteins listed in this category have not so far been reported to bind to PIs.

Identification of PtdIns(4,5)P2-Interacting Proteins from Neomycin Extracts—Our previous data suggests that within the neomycin extracted proteins exits a subset of proteins that bind directly to PIs, in particular PtdIns(4,5)P2. Quantitative lipid pull-down experiments were run to identify specific PtdIns(4,5)P2-interacting proteins from neomycin supernatants (Fig. 4A). Neomycin supernatants obtained from either 12C and 13C-labeled cells were incubated with control beads (beads conjugated to the fatty acid moieties only) or PtdIns(4,5)P2-conjugated beads respectively. Quantitative and statistical analyses revealed the identification of a total of 70 proteins, of which 28 proteins, annotated to the nucleus compartment, were specifically pulled down by PtdIns(4,5)P2 beads. These proteins are listed in Table I. Nineteen of these harbor a K/R motif that represented a twofold enrichment of PtdIns(4,5)P2 interacting proteins containing such motifs compared with noninteracting proteins (Fig. 4B). Functional classification of these proteins pointed to roles in mRNA transcription regulation, mRNA splicing and protein folding (Fig. 4C), the last two categories being over-represented (Fig. 2D) in this dataset.

Topo IIα Associates with PtdIns(4,5)P2—Although previous studies indicated a potential link between the PI pathway and the regulation of Topo IIα activity (54), no data exists to suggest that an interaction between Topo IIα with PIs exists. To demonstrate an interaction between Topo IIα and PIs, neomycin supernatants were dialyzed (removing neomycin) and incubated with control beads or beads conjugated with either PtdIns(4,5)P2 or PtdIns(3,4,5)P3 (Fig. 5A). Proteins bound to lipid beads were eluted and resolved by SDS-PAGE. A positive band for Topo IIα by Western blotting showed the interaction with PtdIns(4,5)P2-conjugated beads, whereas binding to PtdIns(3,4,5)P3-conjugated beads was marginally greater than control beads (Fig 5A). These data demonstrated specificity for binding to PtdIns(4,5)P2. As a control, specific interactions with PtdIns(4,5)P2 or PtdIns(3,4,5)P3 beads were demonstrated by lipid pull-down assays of known proteins harboring PH domains interacting specifically with either PtdIns(4,5)P2 or PtdIns(3,4,5)P3, i.e. PLCδ-PH and GRP1-PH respectively (Fig 5B). The same analysis was performed with purified Topo IIα. In this case, Topo IIα specifically bound to PtdIns(4,5)P2 whereas binding to PtdIns(3,4,5)P3 was at control level (Fig 5C). We also investigated the ability of free PIs to compete with PtdIns(4,5)P2-beads for binding to purified

Fig. 3. GO functional analysis for overrepresented biological processes and molecular functions. Statistically significant overrepresented GO categories for both biological processes (A) and molecular functions (B), as a result of the comparison of the neomycin-extracted protein dataset to the murine nucleome reference data set. Each GO category is represented as the ratio between % neomycin (% of annotated proteins of the whole neomycin set) and % nucleome (% of annotated proteins of the whole nucleome) and are visualized as black bars (complete list). The gray bars represent the statistically significant overrepresented GO categories when the same analysis was done but with only proteins containing K/R motifs, PH and PHD domains as the test dataset (proteins with PI domain). See Supplemental Figs. S2 and S3 for a visualization as hierarchy networks.
Fig. 4. Quantitative and functional analysis of PtdIns(4,5)P₂ pulled down proteins from neomycin extracts. A, Workflow of the quantitative pull-down strategy. ¹³C K/R-labeled and ¹²C K/R-labeled nuclei were incubated with 5 mM neomycin. Displaced proteins were pulled down at equal concentration with control beads (¹²C) or PtdIns(4,5)P₂-conjugated beads (¹³C). Proteins in mixed eluates were resolved by SDS-PAGE, Coomassie stained and trypsin digested. Peptides were analyzed by LC-MS/MS and ¹³C/¹²C ratios were quantified using MSQuant and statistics were determined with StatQuant (42). Seventy proteins with at least two peptides and with significant (p < 0.05) Log₂ ¹³C/¹²C ratios were retained for further analysis. Log₂ > 0.5 represent proteins specifically pulled down by PtdIns(4,5)P₂-beads (listed in Table I). B, Protein classification according to their Log₂ values: proteins with Log₂ < −0.5 (binding to control beads, 26 proteins), Log₂ > 0.5 (binding to PtdIns(4,5)P₂, 34 proteins) and proteins with −0.5 < Log₂ < 0.5 (equal interaction with either control or PI beads, 10 proteins). C, Functional classification of PtdIns(4,5)P₂-interacting proteins according to their biological process. D, Overrepresented biological processes for the PtdIns(4,5)P₂-interacting proteins compared with all proteins annotated to the nucleus compartment (p values: *** p < 0.001).

Topo IIα (Fig. 5D). PtdIns(4,5)P₂ competed for binding of Topo IIα to PtdIns(4,5)P₂ beads in a dose dependent manner.

Examination of the Topo IIα protein sequence highlighted the presence of 7 K/R motifs, all located in the regulatory C-terminal domain (CTD). Sequence alignment of these motifs are shown with other known PtdIns(4,5)P₂ binding proteins with the same K/R motif, such as gelsolin (55), cofilin (56), villin (57), and myristoylated alanine-rich protein kinase C substrate (58) (Supplemental Table S4). To further determine if Topo IIα could interact with PIs, GST fused Topo IIα-CTD protein was purified and used for analysis by SPR (Fig. 5E). GST-proteins with known PI binding affinities were used to validate the assay: The PH domain from PLCδ1 interacted with PtdIns(4,5)P₂, the 2xFYVE domain interacted with PtdIns(3)P and the PHD finger from ING2 interacted with PtdIns(5)P (Supplemental Fig. S4). GST alone did not interact with any of the lipids (Fig 5E). GST-Topo IIα-CTD interacted with PtdIns(4,5)P₂ and PtdIns(5)P (Fig 5E) as well as with PtdIns(3,4,5)P₃ (data not shown). We attempted to demonstrate binding of Topo IIα to liposomes consisting of PIs and phosphatidylcholine in a ratio of 1 to 100. Although we were able to demonstrate interaction of liposomes with the previously characterized PI binding proteins (PH-PLCδ1, 2xFYVE and PH-GRP1), no interaction was seen with the Topo IIα CTD domain or the 2xING2 PHD finger. Why the CTD and PHD finger should only recognize PIs when presented on their own is not clear but may be because of several factors: the properties of the lipids or vesicles used in the assay or the use of isolated domain instead of the full length protein. In addition, it may also be related to the fact that nuclear proteins may not be suited to interacting with PIs in the context of a membrane as this is unlikely to be the physico-chemical nature of PIs in the nucleus.

The ability of PIs to modulate Topo IIα activity in vitro was assessed by kDNA decatenation assays and visualized by agarose electrophoresis (Figs. 5F and 5G). In the absence of the enzyme, the catenated kDNA was restricted from entering the gel (lane 1), whereas the decatenated kDNA resulted in the presence of two processed DNA bands in the presence of
### Table I

**PtdIns(4,5)P2 interacting proteins dataset. Proteins pulled down by PtdIns(4,5)P2, identified with at least 2 peptides, with \( p < 0.05 \) after analysis with StatQuant, and with \( C_{13}/C_{12} \) log2 ratios \( >0.5 \), are indicated in this table.**

| IPI ID     | Gene name | Uniprot   | Ratio  | Log ratio | Ratio STDEV | Number peptides | p value | % protein coverage | Description                                                                 | K/R motif sequence | K/R motif position | GO cell compartment | GO biological process                      | Note                                                                 |
|------------|------------|-----------|--------|-----------|-------------|----------------|---------|-------------------|-----------------------------------------------------------------------------|-------------------|-------------------|-------------------|--------------------------------------------|----------------------------------------------------------------------|
| IPI00399961 | Api5       | O35841    | 1.507  | 0.580     | 0.206       | 6              | 9.84E-04 | 12                | Puteative uncharacterized protein                                           | KGSTKEKR          | 36-43             | Nucleus, cytoplasm | Inhibition of apoptosis                          |                                                                      |
| IPI00319992 | Hspa5      | P20029    | 1.630  | 0.657     | 0.397       | 8              | 0.00225 | 16                | 78 kDa glucose-regulated protein precursor                                  | RRVEKAKR          | 290-298, 280-291 | Nucleus, cytoplasm | Protein folding; Protein complex assembly; Stress response |                                                                      |
| IPI00120691 | Ddx21      | Q9JIK5    | 1.428  | 0.673     | 0.298       | 11             | 2.0E-05  | 18                | Nuclear RNA helicase 2                                                      | KPSEKKTRK         | 24-33             | Nucleus           |                                                                 |                                                                      |
| IPI00225961 | Phgdh      | Q61753    | 1.507  | 0.762     | 0.166       | 4              | 0.0275   | 9                 | 3-phosphoglycerate dehydrogenase                                            | No pattern         |                   |                   |                                                                 |                                                                      |
| IPI00153212 | Ccdc137    | Q8ROK4    | 1.721  | 0.782     | 0.087       | 2              | 0.4999   | 9                 | Charged-coiled-domain-containing protein 137                               | KKLKNKKRKRK       | 78-88             | Nucleus           | Unannotated Amino acid biosynthesis            |                                                                      |
| IPI00121311 | Csd2       | Q61478    | 1.987  | 0.873     | 0.638       | 6              | 0.02036  | 40                | Dihydrolipoamide reductase dehydrogenase                                   | No pattern         |                   |                   | Nucleus, cytoplasm mRNA transcription regulation |                                                                      |
| IPI00222509 | Rcc2       | Q8BK67    | 1.869  | 0.897     | 0.143       | 3              | 0.0838   | 5                 | Regulator of chromosome condensation 2                                     | No pattern         |                   |                   | Nuclear protein translation regulation          | Unannotated Biological process unclassified K/R motif within cell-cell domain 1 |
| IPI00331092 | Rps4x      | P2702     | 1.929  | 0.927     | 0.279       | 5              | 0.00176  | 22                | 4OS ribosomal protein S4, X isoform                                        | KYKLOKVRK         | 120-128           | Cytoplasm         |                                                                 |                                                                      |
| IPI00150684 | Myef2      | Q8CBS54   | 1.928  | 0.933     | 0.248       | 3              | 0.02267  | 5                 | Isoform 2 of Myelin expression factor 2                                     | No pattern         |                   | Nucleus           | mRNA splicing                                            |                                                                      |
| IPI00138301 | Smp         | Q6013J     | 1.555  | 0.947     | 0.284       | 4              | 0.0687   | 23                | SAP domain-containing ribonucleoprotein                                     | RKSELDEKLLK       | 165-176           | Nucleus, cytoplasm | mRNA transcription regulation                    |                                                                      |
| IPI00136883 | Pibp1      | Q7T8R4    | 1.964  | 0.952     | 0.303       | 3              | 0.0321   | 6                 | Polyomavirus TRF binding protein 1                                         | KGFKKFKQKORK      | 481-491           | Nucleus           | mRNA splicing                                            |                                                                      |
| IPI00406118 | Syncrip    | Q7TMK9    | 2.033  | 1.022     | 0.074       | 4              | 0.0001   | 11                | Isoform 2 of Heterogeneous nuclear ribonucleoprotein Q                     | KPDQOQKRKERT      | 406-417           | Nucleus, cytoplasm | mRNA splicing                                            |                                                                      |
| IPI00407130 | Plm2       | P52480    | 2.400  | 1.024     | 0.137       | 3              | 0.0589   | 6                 | Isoform M2 of Pyruvate kinase isozymes M1/M2                               | No pattern         |                   | Nucleus, cytoplasm | Glycolysis                                               |                                                                      |
| IPI00307837 | Eef1a1     | P10126    | 2.072  | 1.048     | 0.100       | 4              | 0.00024  | 9                 | Elongation factor 1-alpha                                                  | No pattern         |                   | Nucleus, cytoplasm | Translational regulation                          |                                                                      |
| IPI00126148 | Mafk       | Q61827    | 2.077  | 1.054     | 0.083       | 2              | 0.3524   | 24                | Transcription factor Mafk                                                  | KPKNKALVKVKK      | 6-15, 46-56       | Nucleus            | mRNA transcription regulation; Cell proliferation and differentiation |                                                                 |                                                                 |
| IPI00410836 | Fen1       | P39749    | 2.177  | 1.122     | 0.013       | 2              | 0.0513   | 5                 | Rap endonuclease 1                                                         | KAKTGGAQKFRF      | 366-378           | Nucleus, cytoplasm | DNA replication; DNA repair                         |                                                                 |                                                                 |
| IPI00110283 | Cct3       | P80318    | 2.217  | 1.125     | 0.319       | 3              | 0.02571  | 3                 | T-complex protein 1 subunit gamma                                           | No pattern         |                   | Nucleus, cytoplasm | Protein folding; Protein complex assembly          |                                                                 |                                                                 |
| IPI00117877 | Matg       | O54790    | 2.221  | 1.140     | 0.215       | 3              | 0.01163  | 17                | Transcription factor Matg                                                  | KGGAKALVKKRR       | 6-15, 46-56       | Nucleus            | mRNA transcription regulation; Cell proliferation and differentiation |                                                                 |                                                                 |
| IPI00420261 | Hmgb1      | P63158    | 2.287  | 1.176     | 0.280       | 3              | 0.01839  | 20                | High mobility group protein B1                                             | KKGVVKAXAKKXX     | 172-185           | Nucleus, cytoplasm | Biological process unclassified mRNA splicing |                                                                 |                                                                 |
| IPI00117288 | Harmapb    | P99020    | 2.272  | 1.182     | 0.089       | 6              | 5.2E-07  | 21                | Heterogeneous nuclear ribonucleoprotein A/B                                 | No pattern         |                   | Nucleus, cytoplasm |                                                                 |                                                                      |
| IPI ID       | Gene name | Uniprot | Ratio | log Ratio | (log) STDEV | Number peptides | p value | % protein coverage | Description                                                                 | K/R motif sequence | K/R motif position | GO cell compartment | GO biological process | Note                                                                 |
|-------------|-----------|---------|-------|-----------|-------------|----------------|---------|--------------------|--------------------------------------------------------------------------------|------------------|-------------------|---------------------|----------------------|----------------------------------------------------------------------|
| IPI00119305 | Pa2g4     | P50580  | 2.326 | 1.218     | 0.016       | 2              | 0.00602 | 4                  | Proliferation-associated protein 2G4                                         | RKTOKKKKKKK      | 363-373           | Nucleus, cytoplasm     | Protein biosynthesis; Cell proliferation and differentiation           | K/R motif within region of interaction with RNA aa 381-375           |
| IPI00116281 | Cct6a     | P80317  | 2.357 | 1.237     | 0.007       | 2              | 0.00247 | 3                  | T-complex protein 1 subunit zeta                                           | No pattern        |                   | Nucleus, cytoplasm     | Protein folding; Protein complex assembly                            |                                                                       |
| IPI00469268 | Cct8      | P42932  | 2.378 | 1.248     | 0.018       | 3              | 0.00139 | 5                  | T-complex protein 1 subunit theta                                           | No pattern        |                   | Nucleus, cytoplasm     | Protein folding; Protein complex assembly                            |                                                                       |
| IPI00230086 | Hrnrnpd   | Q60668  | 2.44  | 1.296     | 0.075       | 2              | 0.0262  | 9                  | Isoform 2 of Heterogeneous nuclear ribonucleoprotein D0                     | No pattern        |                   | Nucleus, cytoplasm     |                                                                 |                                                                       |
| IPI00135686 | Ppib      | P24369  | 2.450 | 1.291     | 0.097       | 3              | 0.00196 | 18                 | Peptidylprolyl isomerase B                                                  | No pattern        |                   | Nucleus, cytoplasm     |                                                                 |                                                                       |
| IPI00462291 | Hmgb2     | P30681  | 2.599 | 1.372     | 0.160       | 3              | 0.00448 | 27                 | High mobility group protein B2                                             | KGDKGKKKKK       | 82-89             | Nucleus, cytoplasm     | Biological process uncategorized                                    | mRNA splicing                                                             |
| IPI00129430 | Sfqp      | Q8VU6   | 2.612 | 1.383     | 0.110       | 2              | 0.03592 | 2                  | Splicing factor, proline- and glutamine-rich                                | No pattern        |                   | Nucleus, cytoplasm     |                                                                 |                                                                       |
| IPI00458683 | Hrnrpu    | Q8VEK3  | 2.676 | 1.401     | 0.251       | 8              | 9.77E-07 | 10                 | Heterogeneous nuclear ribonucleoprotein U                                  | KEELKRR          | 17-24             | Nucleus, cytoplasm     | Other nucleosome, nucleotide and nucleic acid metabolism              | K/R motif within SAP domain aa 8-42                                    |
| IPI00318841 | Eef1g     | Q6D8N0  | 2.840 | 1.506     | 0.047       | 2              | 0.01396 | 6                  | Elongation factor 1-gamma                                                   | No pattern        |                   | Nucleus, cytoplasm     |                                                                 |                                                                       |
| IPI00555069 | Pgi1      | P09411  | 2.862 | 1.513     | 0.150       | 2              | 0.04441 | 8                  | Phosphoglycerate kinase 1                                                  | KLDVKGR          | 11-18             | Nucleus, cytoplasm     |                                                                 |                                                                       |
| IPI00118875 | Eef1d     | P57776  | 2.890 | 1.531     | 0.011       | 3              | 1.58E-05 | 6                  | Eukaryotic translation elongation factor 1 delta isoform a                  | ROYAEKKKKK       | 180-189           | Nucleus, cytoplasm     |                                                                 |                                                                       |
| IPI00230108 | Pdia3     | P27773  | 3.579 | 1.836     | 0.116       | 8              | 7.1E-10  | 19                 | Protein disulfide-isomerase A3 precursor                                    | KKMTSLEGKKK      | 225-234           | Nucleus, cytoplasm     |                                                                 |                                                                       |
| IPI00555023 | Gstp1     | P19157  | 3.643 | 1.864     | 0.082       | 2              | 0.01972 | 12                 | Glutathione S-transferase P 1                                              | No pattern        |                   | Nucleus, cytoplasm     | Unannotated                                                                 | Detoxification                                                            |
| IPI00453827 | Tbc1d24   | Q5UUG6  | 13.07 | 3.703     | 0.168       | 2              | 0.02037 | 6                  | Isoform 2 of TBC1 domain family member 24                                  | RQKIGTVQKQR      | 312-322           | Nucleus, cytoplasm     | Unannotated                                                                 | Biological process uncategorized                                      |
Topo IIα (lane 2), as expected. The addition of PtdIns(3)P, PtdIns(5)P, PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 dramatically reduced the enzyme activity whereas the inhibition was less pronounced with PtdIns(4)P and phosphatidic acid.

**DISCUSSION**

Emerging data point to significant roles for PIs within the context of mammalian nuclei (24, 26–29) and identifying effector proteins for these lipids represents an important first step in deciphering PI-regulated nuclear functions. Currently the most successful methods used to identify effector proteins include the direct affinity purification of PI-binding proteins using lipid-conjugated beads. A prerequisite for the successful application of affinity-bead based enrichment protocols is generating a suitable starting protein extract, i.e. one that has reduced protein complexity whereas maintaining an enriched pool of target proteins (PI-binding proteins in this case). However, affinity-purifying PI effector proteins directly from total nuclear lysates is, at least from a proteomic point of view, fraught with issues of sample complexity and protein dynamic range. The overwhelming presence of abundant polybasic proteins present in the total nuclear extract would exclude or mask the binding of other less abundant bona-fide PI binding proteins by nonspecifically binding to the PI conjugated beads. Furthermore, because the physico-chemical nature of the protein-PI interaction within the nucleus is unclear, we cannot rule out that endogenous PIs remain bound to their effector proteins, thereby competing with the bead-conjugated PI during lipid pull downs. To address these issues, we used neomycin extraction as a means of both reducing sample complexity and enriching for a pool of potential PI-binding proteins devoid of bound PI. Several prior observations suggested that incubating isolated nuclei in the

**Fig. 5. Topo IIα interacts with PtdIns(4,5)P_2.** A, Control (−) and neomycin (+) dialyzed supernatants were incubated with control beads (Cont, only DiC6), PtdIns(4,5)P_2 (P2)- or PtdIns(3,4,5)P_3 (P3)-conjugated beads (pull down). Five percent input and eluates were analyzed by Western blotting using an anti-Topo IIα antibody. B, GST-PLCδ1 PH and GST-GRP1 PH were incubated with PtdIns(4,5)P_2 (P2)- or PtdIns(3,4,5)P_3 (P3)-conjugated beads (pull down). Inputs and eluates were analyzed by Western blotting using an anti-GST antibody. C, EGFP-His-Topo IIα was incubated with control beads or PtdIns(4,5)P_2 (P2)- and PtdIns(3,4,5)P_3 (P3)-conjugated beads (pull down) (D) or with PtdIns(4,5)P_2-conjugated beads in the presence or absence of free lipids. Inputs and eluates were analyzed by Western blotting using an anti-Topo IIα antibody. E, Analysis of binding of GST-TopoIIα-CTD (1 μM) and GST (1 μM), using SPR to PtdIns(3)P (light blue), PtdIns(5)P (dark blue), PtdIns(3,5)P_2 (green) and PtdIns(4,5)P_2 (pink). See also Supplemental Fig. S4. F, Topo IIα activity determined in the absence (−) or presence of free PIs (10 μM) and apparent in the form of decatenated kDNA (indicated with a filled arrow). G, Quantification of Topo IIα activity from 3 independent experiments.

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presence of excess neomycin would produce an extract that is both relatively less complex than a total nuclear lysate and importantly enriched in PI-binding proteins whose PI-binding domain(s) is or are devoid of any endogenous nuclear derived PI. First, neomycin binds PIs with high affinity and immobilized neomycin has been extensively used to purify PIs from tissue and cellular lipid extracts (47, 49, 50). Second, neomycin can compete with PtdIns(4,5)P$_2$ specific antibodies for nuclear PtdIns(4,5)P$_2$ binding sites in intact cell nuclei (24). Third, fluorescently labeled neomycin can bind to PtdIns(4,5)P$_2$ and be used to visualize the lipid in model membrane preparations (59), plasma membrane and nuclear structures (60). Our nano LC-MS/MS data identified prominent proteins, with apparent specificity for the neomycin extract, such as histone H1 and nucleophosmin, two proteins reported to bind PIs (33, 53), substantiating our working hypothesis, such as histone H1 and nucleophosmin, two proteins reported to bind PIs (33, 53), substantiating our working hypothesis. Gel-based comparative analysis however is an inefficient means of determining specificity and we combined our neomycin extraction with SILAC labeling of cells (61) to provide greater sensitivity, throughput and depth of analytical coverage (62). SILAC is a powerful technique, not only for quantitative proteomic analyses, but also as a tool for comparative experiments assessing specificity. SILAC analysis allowed the identification of 349 proteins that were specific for the neomycin extract by our criteria, dramatically increasing the repertoire of neomycin extracted proteins from our initial Coomassie blue gel-staining analysis.

The neomycin extracted proteins included those with authentic PI-binding domains such as dynamin-2 (63) and the PtdIns(3,4,5)P$_3$ binding protein BTK (63). Using either the SMART or Pfam software we also identified proteins that, to our knowledge, have yet to be characterized as PI-binding proteins but which nevertheless have PHD or PH domains within their sequence: Phf8, UHRF1, Fbx111, Skap2 and Arh-GEF2. By far, the majority of the identified proteins possessed at least one K/R motif. Histone H1 for example was identified and has been shown to bind PtdIns(4,5)P$_2$ at the C-terminal domain that contains four K/R-rich motifs (53). All four Histone H1 variants (a,b,c and d) were identified in neomycin extracts. K/R motifs are of particular interest here, especially as they were first described as PtdIns(4,5)P$_2$ binding domains for the actin-binding protein gelsolin (55) and were subsequently extended to include other cytoskeletal binding proteins, such as villin and coflin (56–57). How these cytoskeletal proteins bind PIs is still unclear. It is not known if these interactions occur either as a component of a membrane structure or via a pool of cytosolic PtdIns(4,5)P$_2$ distinct from any membrane structures. This has some similarity within the largely membrane free nuclear matrix and it is intriguing to speculate that a similar mechanism of interaction exists for both nuclear-PI and cytoskeletal-PI interactions via these K/R motifs, within the context of a membrane-free environment. The K/R motifs identified also share some similarities to nuclear localization sequences (NLS): the bipartite NLS sequence of KR-(X$_9$–23)–KKK and K-K/R-X-K/R for monopartite NLS (65). We cannot rule out the possibility that some of these motifs may function as a NLS and have no relevance to nuclear PI metabolism. There are however examples of proteins, such as SAP30L and SAP30, which contain a NLS that has subsequently been shown to function as a binding motif for monophosphoinositides in the context of DNA binding regulation (64), thereby setting a precedent for a multifunctional role for these motifs. Finally the absence of a recognized PI-binding motif does not exclude these candidate proteins from being PI effectors. Nucleophosmin, for example, binds to PtdIns(3,4,5)P$_3$ via several lysine residues within its C terminus in the absence of any characterized PI-binding domain (33). We identified nucleophosmin in our list highlighting the use of neomycin extraction as a non-biased approach for extracting nuclear PI effector proteins.

Cluster analysis of the neomycin specific proteins highlighted several biological processes represented by proteins in these extracts: DNA topology, nucleosome positioning/assembly and DNA packaging, chromatin assembly/disassembly, mRNA processing/RNA splicing and proteins involved in ribosomal related functions (Supplemental Table S2). A growing body of data links nuclear PI metabolism to RNA splicing and mRNA processing events via their colocalization to proteins that define nuclear subdomains called nuclear speckles (34). These domains consist of interchromatin granule clusters, are largely devoid of DNA and have been suggested to be sites for the assembly/storage/modification of predominantly splicing factors that can be released to sites of active transcription. These structures contain snRNPs, mRNA-splicing factors, and a hyperphosphorylated form of RNA polymerase II (66). PIPKs and their product PtdIns(4,5)P$_2$ reorganize identically with speckles, both spatially and temporally (23, 24), suggesting direct interaction of PIPKs with speckle component(s). Several speckle associated proteins involved in RNA splicing were also identified in our neomycin extracts; U2 snRNP A’, U4/U6 snRNP Prp4, SF3A1, U2AF65, DDX5/p68, SF2/ASF and CPSF-100. SF3A1, U2AF65, DDX5/p68, SF2/ASF and CPSF-100 and the canonical poly(A) polymerase (PAP) (68). Both CPSF-73 and CPSF-100 interact directly with the noncanonical Star-PAP, a protein that is not only regulated by...
Protein sequence analysis of Topo II binds to PIs and this interaction localizes Aly to nuclear RNA helicase Bat1/UAP56 that is known to recruit mRNAs to the inhibition of a nuclear PtdIns(5)P2-induced increases in PtdIns(5)P2 levels, in addition perhaps to how this reflects the situation in vivo. We have stated above that it is unlikely that the entire range of proteins extracted by neomycin represent PI effector proteins. Neomycin extracted proteins therefore represent an ideal preparation from which to affinity-purify PI-effector proteins using specific lipid conjugated matrices, avoiding the issues of sample complexity and dynamic range addressed previously. Probing SILAC labeled neomycin extracts with PtdIns(4,5)P2 conjugated beads resulted in the identification of 28 novel, nuclear PtdIns(4,5)P2-interacting proteins. More than half of these proteins were characterized by the presence of K/R motifs and more importantly, the proportion of proteins presenting such motifs was twofold increased in PtdIns(4,5)P2-interacting proteins compared with noninteracting proteins, which may suggest a mode of interaction for PI binding proteins localized in the nucleus.

In summary, we have shown that neomycin displaced over 300 proteins from isolated nuclei, and almost half of these harbor potential PI binding domains. These PI binding domains were mostly represented by unstructured positively charged K/R motifs and these motifs may represent predominant interaction sites for nuclear proteins with PI. Some of these proteins have been implicated in PI binding but many proteins with no previous history of PI binding were also identified. Furthermore, cluster analysis for biological functions highlighted several categories such as mRNA processing and splicing, DNA topological changes and chromatin assembly. To this end our proteomic methods have also enabled us to identify nuclear PtdIns(4,5)P2 interacting proteins that indeed provide an important resource on which to base future investigations. This work not only represents a global approach to identifying nuclear PI effector proteins, it also represents a global approach to identify the array of nuclear functions dependent on nuclear PI turnover.

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