A substrate-trapping strategy for protein phosphatase PP1 holoenzymes using hypoactive subunit fusions

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The protein Ser/Thr phosphatase PP1 catalyzes an important fraction of protein dephosphorylation events and forms highly specific holoenzymes through an association with regulatory interactors of protein phosphatase one (RIPPOs). The functional characterization of individual PP1 holoenzymes is hampered by the lack of straightforward strategies for substrate mapping. Because efficient substrate recruitment often involves binding to both PP1 and its associated RIPPO, here we examined whether PP1–RIPPO fusions can be used to trap substrates for further analysis. Fusions of an inactive point mutant of PP1 and either of four tested RIPPOs accumulated in HEK293T cells with their associated substrates and were co-immunoprecipitated for subsequent identification of the substrates by immunoblotting or MS analysis. Hypoactive fusions were also used to study RIPPOs themselves as substrates for associated PP1. In contrast, substrate trapping was rarely detected with active PP1–RIPPO fusions or with nonfused PP1 or RIPPO subunits. Our results suggest that hypoactive fusions of PP1 subunits represent an easy-to-use tool for substrate identification of individual holoenzymes.

Understanding the biological function of enzymes requires knowledge of their physiological substrates. Enzyme substrate mapping can be very challenging but is sometimes facilitated by insights into the catalytic mechanism. Prime examples are protein–tyrosine phosphatases, which have a catalytic-site cysteine that forms a covalent thiophosphoryl intermediate during the first catalytic step that is hydrolyzed during the second step of catalysis (1, 2). Replacement of the active-site cysteine of protein–tyrosine phosphatases by a serine blocks the second catalytic step and results in the accumulation of the covalent catalytic intermediate, which can be immunoprecipitated for subsequent identification of the associated substrate. The same approach is not possible for substrate mapping of protein–Ser/Thr phosphatases because they hydrolyze phosphate esters in a single step and do not form a covalent catalytic intermediate (3). However, protein–Ser/Thr phosphatases often contain one or two noncatalytic subunits that also contribute to substrate recruitment. For example, protein–Ser/Thr phosphatase PP1, a member of the phosphoprotein phosphatase (PPP)2 family, forms highly selective holoenzymes through association with a broad spectrum of regulatory interactors of protein phosphatase one (RIPPOs). The latter proteins are also known as PP1-interacting proteins (PIPs), but this acronym can be confusing as it is also commonly used to denote phosphatidylinositol phosphates. Substrate recruitment by PP1 involves docking of a phospho-Ser/Thr at the active site but may also include additional substrate interactions via Y-shaped surface grooves that emanate from the active site (4–7). In addition, many RIPPOs harbor a low-affinity binding site(s) for a subset of PP1 substrates, which, together with substrate recruitment via the catalytic subunit, creates a selective and high-affinity substrate binding interface. For example, nuclear inhibitor of PP1 (NIPP1) contains a forkhead-associated (FHA) domain that recruits PP1 substrates phosphorylated at phospho-threonine-proline (pTP) dipeptide motifs (8, 9). Other RIPPOs with established substrate-binding domains include RepoMan (recruits PP1 onto mitotic chromatin at anaphase), which contains a histone docking site (10); MYPT1 (myosin phosphatase–targeting subunit 1), which has binding sites for the PP1 substrates myosin (11) and polo kinase 1 (PLK1) (12); and PINS (PP1 nuclear targeting subunit), which mediates the recruitment of RNA polymerase II as a substrate for associated PP1 (13, 14).

The notion that both PP1 and RIPPOs contribute to substrate recruitment prompted us to explore PP1–RIPPO fusions as tools to trap substrates in a noncovalent manner. Because substrates dissociate from PP1 upon dephosphorylation, we also examined whether substrate trapping can be enhanced by the use of PP1–RIPPO fusions with an inactive PP1 moiety. Here we report that inactive PP1–RIPPO fusions accumulate in the cell with their associated substrates and can be immunoprecipitated for subsequent identification of these substrates.

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This article contains Figs. S1–S4 and Tables S1–S3.

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**Results and discussion**

**Substrate trapping with hypoactive fusions of PP1 and NIPP1**

We first transiently transfected HEK293T cells with EGFP-tagged fusions of PP1 and NIPP1 (Fig. 1A). In addition to the WT fusion (PP1–NIPP1), we used fusions with an inactive catalytic moiety (PP1m–NIPP1), a substrate-binding mutant of NIPP1 (PP1–NIPP1m), or a combination of both mutations (PP1m–NIPP1m). PP1m has a mutated metal-coordinating residue in the active site (D64A), which strongly reduces the $V_{\text{max}}$ of PP1 but has no effect on the $K_m$ (15). NIPP1m is mutated in the substrate-binding loop of the FHA domain (S68A/R69A/V70A/H71A), which abolishes substrate recruitment (9, 16). All PP1–NIPP1 fusions accumulated in the nucleus (Fig. S1), as described previously for stably expressed PP1–NIPP1 fusions in HeLa cells (17), and the endogenous PP1:NIPP1 holoenzyme (18, 19). EGFP traps of the PP1–NIPP1 and PP1–NIPP1m fusions showed no spontaneous phosphatase activity with glycogen phosphorylase $a$ as substrate (Fig. 1B). However, a phosphorylase phosphatase activity was revealed by prior trypsinolysis, which releases the fully active catalytic core of PP1 (20). This demonstrated that PP1 was catalytically active in these fusions but completely inhibited by NIPP1 toward PP1 substrates that do not bind to the FHA domain of NIPP1, as observed previously for these fusions in HeLa cells (17), and in vitro reconstituted PP1:NIPP1 (21). As expected, the PP1m–NIPP1 and PP1m–NIPP1m fusions were largely inactive both before and after trypsinolysis.

We proceeded to examine the ability of the PP1–NIPP1 fusions to trap SAP155, an established NIPP1 FHA ligand and substrate of PP1:NIPP1 (22, 23). SAP155 co-precipitated with PP1m–NIPP1 but barely so with PP1–NIPP1 or PP1m–NIPP1m (Fig. 1C). This demonstrates that the trapping of SAP155 is only efficient when NIPP1 has a functional FHA domain and fused to inactive PP1. As expected, SAP155 trapped by PP1m–NIPP1 was phosphorylated on FHA-binding phospho-TP dipeptide motifs (Fig. 1C), as detected with a largely phospho-epitope–specific pan-pTP antibody (Fig. S2A). Conversely, immunoprecipitation of endogenous SAP155 resulted in co-precipitation of ectopically expressed PP1m–NIPP1 (Fig. 1D). However, co-precipitation with PP1–NIPP1 and PP1m–NIPP1m was much less pronounced. Interestingly, SAP155 phosphorylation at TP dipeptide motifs was strongly reduced after the expression of PP1–NIPP1 but was maintained with the hypoactive fusions, consistent with SAP155 being a substrate for PP1–NIPP1 (23). We also found that the interaction of SAP155 with PP1m–NIPP1 was decreased after prior incubation of the lysates with $\lambda$ phosphatase to dephosphorylate SAP155 (Fig. 1E), confirming that the recruitment of SAP155 as a substrate is phosphorylation-dependent (16). Finally, we compared the substrate-trapping abilities of PP1m–NIPP1, PP1m, NIPP1, and a previously described NIPP1 mutant (199RVT$^{203} \rightarrow$ RATA) with a disrupted PP1-binding domain (24) (Fig. 1F). PP1m–NIPP1 co-precipitated more SAP155 than PP1m, NIPP1, or NIPP1–RATA, confirming that efficient substrate trapping depends on substrate binding sites of both NIPP1 and PP1 as well as an inactive catalytic moiety. The latter finding indicates that the docking of a phosphate group at the active site, which is maintained when the catalytic moiety is inactive, contributes significantly to the overall substrate-binding affinity.

**Mapping of phosphorylation sites of SAP155**

Although SAP155 is a known substrate of PP1:NIPP1 (23), the targeted phosphorylation sites have not yet been identified. We compared the phosphorylation status of SAP155 that co-precipitated with PP1m–NIPP1 and PP1–NIPP1 by MS (Fig. 2, A and B). The amount of SAP155 that co-precipitated with PP1m–NIPP1 was 4.9 times higher than that with PP1–NIPP1. However, even when corrected for the same amount of SAP155, it was hyperphosphorylated at 11 sites in the PP1m–NIPP1 traps compared with its phosphorylation status in PP1–NIPP1 traps (Fig. 2, A and B, and Table S1). All hyperphosphorylated sites were pThr residues followed by a Pro, and all but one of these sites were mapped to the TP dipeptide–enriched NIPP1-binding domain of SAP155 (residues 223–437) (22). To independently validate hyperphosphorylation of SAP155 at pTP motifs, we used a commercially available antibody to detect pT$^{315}$. This antibody was specific, as it did not recognize the T313D mutant of SAP155 (Fig. S2B). Using the latter antibody, SAP155 associated with PP1m–NIPP1 was found to be strongly phosphorylated at Thr$^{313}$ (Fig. 2C). In contrast, the much smaller amount of SAP155 associated with PP1–NIPP1 was not detectably phosphorylated at this site. We also noted that the phosphorylation of SAP155 at Thr$^{313}$ in cell lysates was increased by the expression of PP1m–NIPP1 or PP1m–NIPP1m, indicating that these hypoactive fusions hampered SAP155 dephosphorylation at Thr$^{313}$ (Fig. 2C). A SAP155 fragment (residues 1–491) in vitro phosphorylated by cyclinA/CDK2 was dephosphorylated at Thr$^{313}$ by purified PP1 (Fig. 2D). Immunoprecipitated EGFP-tagged PP1–NIPP1 also dephosphorylated this SAP155 fragment, but the dephosphorylation rate was strongly increased by trypsinolysis of NIPP1. This is in accordance with previous data showing that full-length NIPP1 restrains PP1 under basal circumstances (9, 21), even toward FHA ligands, hinting at the existence of an in vivo activation mechanism of PP1:NIPP1. Collectively, our data strongly indicate that pTP dipeptide motifs of SAP155, including pT$^{315}$, are substrates for dephosphorylation by PP1:NIPP1.

**Substrate trapping with other PP1–RIPPO fusions**

Next we examined the substrate-trapping capabilities of (in)active PP1 fused to other RIPPOs, i.e. RepoMan, PNUTS or MYPT1 (Fig. 3A), after their transient expression in HEK293T cells. All examined fusions showed the expected subcellular distribution: a nuclear enrichment for PNUTS and RepoMan but a more diffuse nucleocytoplasmic distribution and plasma membrane association for MYPT1 (Fig. S3). In addition, the active fusions displayed phosphorylase phosphatase activity, but only after prior trypsinolysis (Fig. 3B), showing that PP1 was properly folded but functionally restrained by the associated RIPPO. As expected, the fusions with PP1m showed a much lower activity toward glycogen phosphorylase $a$. Using a largely phospho-epitope–selective Ser/Thr antibody (Fig. S2C), all tested hypoactive PP1 fusions co-precipitated with phosphoproteins to a larger extent than detected with the correspond-
PP1 substrate trapping

A. Flexible Linker

PP1-NPP1
EGFP PP1γ NIPP1

PP1m-NPP1
EGFP PP1γ NIPP1

PP1-NIPP1m
EGFP PP1γ NIPP1

PP1m-NIPP1m
EGFP PP1γ NIPP1

B. PP Activity (% of PP1-NIPP1 + Trypsin)

No Trypsin

Trypsin

EGFP

115 kDa

C.

Ct: IgG

IP: SAP155

SAP155

pTP

Fusions (Ab: EGFP)

D.

E.

λ-phosphatase

- + - + - +

EGFP

SAP155

pTP

F.

EGFP

SAP155

pTP

INPUT

GAPDH
ing active fusions (Fig. 3C). For RepoMan, the differences were even larger with lysates from prometaphase-arrested cells, consistent with previous findings that PP1:RepoMan is particularly active during mitosis (10, 25). We also confirmed these data by detecting established phospho-substrates with the hypophosphorytic fusions, i.e. histone H3 at Thr\(^3\) for PP1m–RepoMan, Ser\(^\alpha\) of the heptapep peptides in the carboxyterminal domain of the largest subunit of RNA polymerase II for PP1m–PNUTS and Thr\(^{210}\) of PLK1 for PP1m–MYPT1 (Fig. 3C). For RNA polymerase II and PLK1, increased phosphorylation correlated with increased binding to PP1m–MYPT1, indicating that their efficient recruitment as a substrate is phosphorylation-dependent.

**Identification of novel substrates of PP1:RepoMan**

We have subsequently identified candidate substrates of PP1:RepoMan through mass spectrometric mapping of the phosphopeptides that are associated with PP1m–RepoMan and PP1–RepoMan, trapped from mitotically arrested cells. Table S2 shows a selection of 81 phosphopeptides, excluding those derived from RepoMan itself (see next section), that were preferentially associated with PP1m–RepoMan (phosphopeptide abundance ratio PP1m–RepoMan/PP1–RepoMan > 1.5). Fig. 4A lists the top hits of 21 phosphopeptides from 12 distinct polypeptides. They mainly comprise chromatin-associated proteins, in accordance with the similar subcellular distribution of RepoMan (25, 26). PP1:RepoMan is already known to dephosphorylate histone H3 (26, 27), and the data shown in Fig. 4A suggest that PP1–RepoMan may also be involved in the dephosphorylation of other histones (Thr\(^{129}\) of macro-H2A1, Ser\(^{\beta7}\) of H1.3). Other chromatin-associated candidate substrates included HMG1A (high-mobility group HMG-I/HMG-Y), MKI67 (marker of proliferation Ki-67), PDS5B (sister chromatid cohesion protein PDS5 homolog B), and MYBBP1A (MYB-binding protein 1A).

We have experimentally validated two proteins from Fig. 4A as substrates of PP1:RepoMan, the PP2A regulatory subunit B56\(\delta\)/PPP2R5D and PP1\(\gamma\), as they were the only ones for which phospho-epitope–specific antibodies were available. B56 is an established mitotic interactor of RepoMan (25) but is not known to be a substrate of PP1–RepoMan. We found that human B56\(\delta\) preferentially co-precipitated with the hypophosphorytic PP1m–RepoMan fusion and confirmed by immunoblotting with a phospho-epitope–specific antibody (28) that the trapped B56\(\delta\) was phosphorylated at Ser\(^{573}\) (Fig. 4B). Quantification of the blots revealed that the phosphorylation of B56 at Ser\(^{573}\) in the PP1m–RepoMan traps was more increased (5-fold) than the amount of B56 (2.8-fold). Ser\(^{573}\) of B56\(\delta\) was dephosphorylated by PP1 purified from rabbit skeletal muscle as well as by PP1–RepoMan (Fig. 4C). Because the phosphorylation of B56\(\delta\) at Ser\(^{573}\) by protein kinase A contributes to the activation of PP2A (28, 29), these data suggest that RepoMan-associated PP1 inactivates the pool of PP2A–B56 that is bound to RepoMan.

A fraction of PP1 is phosphorylated during mitosis at its C terminus by protein kinase CDK1 (Thr\(^{11}\) for PP1\(\gamma\)) but autodephosphorylated when the activity of CDK1 drops in anaphase (30, 31). However, it is not known which PP1 holoenzymes are regulated by mitotic phosphorylation of the catalytic subunit. We found that the PP1\(\gamma\) moiety of the PP1m–RepoMan fusion was hyperphosphorylated at Thr\(^{11}\) in nocodazole-arrested cells (Fig. 4D), indicating that the RepoMan-associated pool of PP1 is a substrate for CDK1 and autodephosphorylation in cis.

**RIPPos as substrates for associated PP1**

Often RIPPos are themselves substrates for associated PP1 (32). For example, RepoMan has numerous mitotic CDK1 phosphorylation sites, and at least some of these are dephosphorylated by associated PP1 (25, 26). A mass spectrometric analysis revealed that the RepoMan moiety of the PP1m–RepoMan fusion was hyperphosphorylated in prometaphase-arrested cells at 21 sites, mainly mapped to the N-terminal and C-terminal thirds of RepoMan (Fig. 5, A and B). Of these, six sites were Ser-Pro consensus sites for CDK1. These include N-terminal phosphorylation sites (Ser\(^{258}\), Ser\(^{259}\), Ser\(^{260}\), and Ser\(^{261}\)) that control the binding of importin \(\beta\) and are known substrates for RepoMan-associated PP1 (25, 26). However, we also mapped two novel CDK phosphorylation sites to the C-terminal half of RepoMan (Ser\(^{710}\) and Ser\(^{756}\)). Mitotic hyperphosphorylation of PP1m–RepoMan at CDK sites was confirmed with MPM2 phospho-epitope–specific antibodies (Fig. 5C), and these sites were dephosphorylated with purified PP1 (Fig. 5D). In addition to the six putative CDK1 sites, the RepoMan moiety of PP1m–RepoMan was also hyperphosphorylated in prometaphase-arrested cells at 15 other sites (Fig. 5, A and B), but the involved kinases and their function are unknown. However, two of these sites (Ser\(^{808}\) and Ser\(^{960}\)) are followed by a Glu and represent consensus sites for phosphorylation by the RepoMan interactor.

**Figure 1. Substrate trapping by PP1–NIPP1 fusions.** A, schematic of EGFP-tagged PP1–NIPP1 fusions. B, HEK293T cells were transiently transfected with plasmids encoding the indicated EGFP-tagged proteins. Spontaneous and trypsin-revealed glycogen phosphorylase phosphatase (4B) were dephosphorylated with purified PP1. C, MPM2- and MPM2c-tagged PP1–NIPP1 fusions were dephosphorylated with PP1 purified from rabbit skeletal muscle as well as by PP1–RepoMan (Fig. 4C). Because the phosphorylation of B56\(\delta\) at Ser\(^{573}\) by protein kinase A contributes to the activation of PP2A (28, 29), these data suggest that RepoMan-associated PP1 inactivates the pool of PP2A–B56 that is bound to RepoMan.
protein kinase ataxia telangiectasia–mutated (ATM) (33). Collectively, our data confirm, in an independent manner, that RepoMan is itself a substrate for associated PP1.

Conclusions
We have shown here that all four examined fusions of inactive PP1 and RIPPOs accumulated in the cell with their associated substrates (Fig. 6). The interaction of PP1m–RIPPO fusions with their substrates remained intact during immunoprecipitation, which allowed the identification of these substrates by immunoblotting or MS. The superior substrate-trapping capability of hypoactive fusions compared with active fusions or nonfused subunits can be explained by the combination of substrate-binding sites on PP1 and RIP-

### Table: SAP155 Phosphopeptide Sequence and Abundance Ratio

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<tr>
<th>Phosphopeptide Sequence</th>
<th>Phosphopeptide Abundance Ratio</th>
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<tbody>
<tr>
<td>WQADQTPGApTPK</td>
<td>5</td>
</tr>
<tr>
<td>KLSWVDAEPHpTPSLR</td>
<td>3</td>
</tr>
<tr>
<td>KLSWVDAEPHpTPSLR</td>
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</tr>
<tr>
<td>LSSWDAEPHpTPGHTPSLR</td>
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<tr>
<td>WDTPSHpTPAGATPGR</td>
<td>3</td>
</tr>
<tr>
<td>WDpTPSHpTPAGATPGR</td>
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</tr>
<tr>
<td>GdpTPGHPHGAGTASSAR</td>
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<tr>
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<tr>
<td>DTPGHGWAEpTRP</td>
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</tr>
<tr>
<td>GGDSIGePpppGASK</td>
<td>6</td>
</tr>
<tr>
<td>KLTApPTPLGGMTGFHMQEDR</td>
<td>110</td>
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</table>

**Figure 2.** pTP sites of SAP155 are substrates for PP1–NIPP1. A, sequence of the SAP155-derived phosphopeptides that were preferentially associated with EGFP-trapped PP1m–NIPP1, as identified by MS. The phosphopeptide abundance ratio of SAP155 associated with PP1m–NIPP1 over PP1–NIPP1 was corrected for the same SAP155 protein abundance under both conditions. *B*, schematic of SAP155 with its N-terminal TP dipeptide–enriched NIPP1-binding domain (blue) and its C-terminal heat repeats (green). TP dipeptide motifs are indicated by yellow vertical lines and the TP sites that were hyperphosphorylated in PP1m–NIPP1-traps by blue vertical lines. *C*, lysates from HEK293T cells expressing the indicated EGFP-tagged proteins were subjected to EGFP trapping. The level of total SAP155 and phospho-SAP155 was quantified in lysates and EGFP traps by immunoblotting with SAP155 and pThr313-specific SAP155 antibodies, respectively. GAPDH and EGFP served as loading controls for the total lysates (INPUT) and EGFP traps, respectively. The data are representative of three independent experiments. *D*, bacterially expressed His-SAP155-(1–491) was purified, in vitro phosphorylated with CDK2/CyclinA, and subsequently in vitro dephosphorylated with PP1 (300 nM) from rabbit skeletal muscle or with EGFP-tagged PP1–NIPP1 fusion trapped from HEK293T cell lysates. For the three last conditions, EGFP traps were pretreated with trypsin (+H11001), and trypsinolysis during the phosphatase assay was prevented by addition of soybean trypsin inhibitor. The total and phospho levels of His-SAP155-(1–491) were detected by immunoblotting using SAP155 and pThr313-specific SAP155 antibodies, respectively. The data are representative of three independent experiments. Ctr, control.
POs into a high-affinity binding interface and the inability to dephosphorylate these substrates. The deficient substrate dephosphorylation furthermore explains why inactive fusions can also be used to study RIPPOs as substrates for associated PP1.

We have explored here the use of hypoactive fusions for substrate trapping of PP1 holoenzymes. However, we speculate that this approach also holds great potential for substrate mapping of other multimeric protein Ser/Thr phosphatases.

**Experimental procedures**

The following antibodies were used: anti-GFP (Santa Cruz Biotechnology, sc-8334, Dallas, TX), anti-phospho-threonine-proline (Cell Signaling Technology, 9391, Danvers, MA), anti-SAP155 (MBL, D221-3, Woburn, MA), anti-phospho-SAP155 (Thr213; Cell Signaling Technology, 25090), anti-GAPDH (Cell Signaling Technology, 2118), anti-phospho-serine/threonine (BD Biosciences, 612548, San Jose, CA), anti-phospho-histone H3 (Thr3; Upstate; Uptis, 07-424, Darmstadt, Germany), anti-histone H3 (Sigma-Aldrich, h0164, St. Louis, MO), anti-phospho-RNA polymerase II (Ser5; Abcam, ab5131, Cambridge, UK), anti-RNA polymerase II (Santa Cruz Biotechnology, sc56767), anti-phospho-PLK1 (Thr310; Cell Signaling Technology, 5472), anti-PLK1 (Cell Signaling Technology, 4513), anti-phospho-Ser/Thr-Pro MPM-2 (Merck Millpore, 05-368, Kenilworth, NJ), anti-PPP1R8/NIPP1 (Sigma-Aldrich, HPA027452), anti-B56δ-phospho-Ser\(^{73}\) (a kind gift from Dr. Angus Nairn and Dr. Jung Ahn from Yale University and Ewha Womans University College of Medicine, respectively), anti-B56δ (Bethyl Laboratories, A301-100A, Montgomery, TX), anti-PP1 (Thr320; Cell Signaling Technology, 2581, Danvers, MA), polyclonal swine anti-rabbit IgG/HRP (Dako, P0217, Santa Clara, CA), and polyclonal rabbit anti-mouse IgG/HRP (Dako, P0260). The specificity of the antibodies used for the detection of SAP155-pThr\(^{213}\), pan phospho-Thr-Pro, and pan phospho-Ser/Thr was validated separately (Fig. S2). All other antibodies have been validated in multiple previous studies.

**Phosphatase (sc-200312)** was purchased from Santa Cruz Biotechnology. The PP1–RIPPO fusions were generated as described previously for PP1–NIPP1 (17). The construct for bacterial expression of polyhistidine-tagged EGFP-SAP155-(1–491) was generated by subcloning of EGFP in the previously described His-SAP155-(1–491) construct (22). mCherry-tagged SAP155 (WT) and SAP155-T313D were cloned from the pCDNA3.1-FLAG-SF3B1(WT) plasmid purchased from Addgene (82576). EGFP-tagged B56δ was cloned from the pCEP-4HA B56δ plasmid purchased from Addgene (14536).

**Cell culture and imaging**

HEK293T cells (ATCC, Molsheim, France) were cultured in Dulbecco’s modified Eagle’s medium with high glucose (4,500 mg/liter glucose, Sigma-Aldrich) containing 10% fetal calf serum (Sigma-Aldrich), 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfections were performed with Genius reagent (Westburg, Leusden, The Netherlands). Prometaphase arrest was induced by consecutive treatments with 20 mM thymidine (24 h), release for 3 h, and incubation with 100 ng/ml nocodazole (Sigma-Aldrich) for 15 h. For immunofluorescence imaging, HEK293T cells grown on polylysine-coated coverslips in a 24-well chamber were transfected with the indicated plasmids for 24 h and fixed with 4% paraformaldehyde. 4,6-di-aminido-2-phenylindole was used to stain DNA. Confocal images were acquired with a Leica TCS SPE laser-scanning confocal system.

**Biochemical techniques**

EGFP-trapping assays were performed as described previously (34). 1 × 10\(^5\) HEK293T cells were seeded in a 15-cm plate and transfected with 5 μg of plasmids. After 48 h, the cells were lysed on ice for 30 min with modified radioimmuneprecipitation assay lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM Na\(_2\)EDTA, 2H\(_2\)O, 1% NP40, and 1% sodium deoxycholate) supplemented with protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, and 5 μM leupeptin) and phosphatase inhibitors (25 mM NaF, 50 mM β-glycerophosphate, 0.5 μM microcystin-LR, and 1 mM sodium orthovanadate). Centrifugation of the lysates (10 min at 3,000 × g) yielded a supernatant (S1) and pellet. The pellets were washed twice with 50 mM Tris (pH 8.0) and incubated for 20 min at 37 °C in a shaking incubator in the presence of micrococcal nuclease (300 units/ml) treatment (S2). The S1 and S2 fractions were combined and precleared with 30 μl of BSA beads for 1 h at 4 °C, followed by incubation with 25 μl of GFP-Trap beads (1:1 suspension) for 2–3 h at 4 °C. The beads were washed four to six times with Tris-buffered saline supplemented with 0.1% Triton X-100 and 0.25% NP-40 and subjected to immunoblotting.

Phosphorylase phosphatase assays were performed as described previously (17). For in vitro dephosphorylation with λ phosphatase (Fig. 1E), cell lysates were incubated with or without λ phosphatase (1.6 units/μl of lysate) for 30 min at 30 °C before EGFP trapping. Bacterially expressed His-EGFP-

![Figure 3. Substrate trapping by (in)active PP1 fusions of RepoMan, PNUTS, and MYPT1. A, schematic of EGFP-tagged PP1–RIPPO fusions. B, HEK293T cells were transfected with plasmids encoding the indicated EGFP-tagged PP1–RIPPO fusions. Spontaneous and trypsin-revealed glycogen phosphorylase phosphorylation assays were assayed in the indicated EGFP traps. Trypsinolysis during the phosphatase assay was prevented by addition of soybean trypsin inhibitor. Data are means ± S.D. of three independent experiments (triangles indicate individual data points). The level of EGFP-trapped proteins was verified in each experiment by immunoblotting using EGFP antibodies, and a representative image is shown in the bottom panel. ns, not significant (paired Student’s t test); **, p < 0.01; ***, p < 0.001. C, lysates from asynchronous or prometaphase-arrested HEK293T cells expressing the indicated EGFP-tagged fusions were subjected to EGFP trapping. The global protein phosphorylation status in the traps was visualized by immunoblotting with a pan-phospho-Ser/Thr antibody. Also shown are phosphorylation of histone H3 at Thr3 (H3T3-ph), the heptapeptide repeats of the carboxyterminal domain of the largest subunit of RNA polymerase II at Ser\(^5\) (RNAII-S5ph), and PLK1 at Thr\(^{210}\) (PLK1-T210ph), as well as the total level of these proteins in the EGFP traps. The blots are representative of three independent experiments.](15158_J._Biol._Chem._(2018)_293(39)_15152–15162)(1)}
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**Figure 4. Validation of novel substrates of PP1–RepoMan.** A, sequence of phosphopeptides from proteins that were associated with EGFP-trapped PP1m–RepoMan and PP1–RepoMan, as identified by MS. The traps were from lysates of prometaphase-arrested HEK293T cells. The phosphopeptide abundance ratio of each phosphopeptide associated with PP1m–RepoMan over PP1–RepoMan was corrected for the same protein abundance under both conditions. B, lysates from prometaphase-arrested HEK293T cells expressing the indicated EGFP-tagged proteins were subjected to EGFP trapping. The level of total PP2A-B56δ and phospho-PP2A-B56δ was quantified in EGFP traps by immunoblotting using EGFP and pSer573-specific PP2A-B56δ antibodies, respectively. The blots are representative of three independent experiments. C, lysates of HEK293T cells (4–8 mg of protein) transfected with plasmids encoding EGFP–PP1m–RepoMan or EGFP–PP2A-B56δ were subjected to EGFP trapping. EGFP–PP2A-B56δ associated with the EGFP trap was in vitro phosphorylated with protein kinase A and subsequently in vitro dephosphorylated with PP1 (300 nM) purified from rabbit skeletal muscle or with EGFP–PP1–RepoMan trapped from lysates of transfected HEK293T cells. The level of total PP2A-B56δ and phospho-PP2A-B56δ was quantified by immunoblotting using EGFP and pSer573-specific PP2A-B56δ antibodies, respectively. The blots are representative of three independent experiments. Ctr, control. D, lysates from prometaphase-arrested HEK293T cells expressing the indicated EGFP-tagged fusions were subjected to EGFP trapping. Phosphorylation of the PP1 γ moiety at Thr311 was detected by immunoblotting with a phospho-epitope–specific antibody. The blot also shows phosphorylation of PP1 γ in a degradation product of the hypoactive fusion (asterisk).

SAP155–(1–491) was purified and in vitro phosphorylated with CDK2/CyclinA as described previously (35). His-EGFP-SAP155–(1–491), EGFP–PP1m–RepoMan, and EGFP–B56δ trapped from HEK293T cell lysates were dephosphorylated with PP1 from rabbit skeletal muscle at 30 °C before SDS-PAGE and immunoblotting.
Figure 5. RepoMan is a substrate for associated PP1. A, sequence of RepoMan-derived phosphopeptides as identified by MS in EGFP-trapped PP1m–RepoMan and PP1–RepoMan. The RepoMan phosphopeptide abundance ratio in PP1m–RepoMan over PP1–RepoMan was corrected for the same RepoMan protein abundance under both conditions. 1P/2P refers to phosphorylations whose phosphorylated residue could not be assigned. B, schematic of the sites hyperphosphorylated at the RepoMan moiety of PP1m–RepoMan. Putative CDK sites are indicated in red, and other hyperphosphorylated sites are shown in blue. The PP1-binding domain is indicated. C, EGFP traps were performed in prometaphase-arrested HEK293T cells expressing the indicated EGFP-tagged fusions and analyzed by immunoblotting with the MPM2 antibody, which recognizes CDK phospho-sites. Equal loading of the fusions was verified by immunoblotting using EGFP antibodies. The data are representative of two independent experiments. D, an EGFP trap of PP1m–RepoMan from prometaphase-arrested HEK293T cells was incubated at 30 °C for the indicated time with PP1 (600 nM) from rabbit skeletal muscle. The level of EGFP–PP1m–RepoMan and its phosphorylation at CDK phospho-sites was analyzed by immunoblotting with EGFP and MPM2 antibodies, respectively. The data are representative of three independent experiments.

Figure 6. Model of the substrate-trapping strategy by PP1–RIPPO fusions. A, free PP1 or RIPPOs bind with low affinity to (a subset of) substrates (SUB). B, PP1–RIPPO holoenzymes or PP1–RIPPO fusions combine multiple substrate-binding sites to create a high-affinity interaction interface. However, the high-affinity interaction with substrates is only transient and is disrupted upon dephosphorylation of the substrate. C, PP1m–RIPPO fusions bind with high affinity to their substrates. Because the catalytic moiety is inactive (PP1m), the interaction with substrates is prolonged, enabling their co-immunoprecipitation with the fusions.
Mass spectrometry analysis

EGFP traps (30 μl of nanobody EGFP beads) of EGFP–PP1(m)–NIPP1 or EGFP–PP1(m)–RepoMan from lysates of asynchronous or prometaphase-arrested HEK293T cells, respectively, were subjected to overnight on-bead trypsin digestion (2 μg of trypsin at 37 °C in a total of 60 μl containing 200 mM ammonium bicarbonate and 5% acetonitrile). The resulting peptides were desalted and subjected to phospho-peptide enrichment on IMAC beads (PHOS-Select iron affinity gel, Sigma). The flow-through fraction of IMAC was also collected and analyzed to gauge the differential phosphorylation status of SAP155 in EGFP–PP1m–NIPP1 versus EGFP–PP1–NIPP1 traps or of RepoMan and other phosphoproteins in EGFP–PP1m–RepoMan versus EGFP–PP1–RepoMan traps. Subsequently, the peptides were desalted with C18 ZipTip pipette tips (Millipore) and subjected to high-resolution LC-MS/MS using an Ultimate 3000 Nano Ultra High Pressure Chromatography (UPLC) system interfaced with a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer via an EASY-spray (C-18, 15 cm) column (Thermo Fisher Scientific). Peptides were identified by MASCOT (Matrix Science) using UniProt Homo sapiens (169,779 sequences) as a database. The following MASCOT search parameters were used: Trypsin/P, oxidation (M), phosphorylation (STY). Three missed cleavages were allowed for trypsin digestion. Peptide tolerance was set at 10 ppm for MS and at 20 milli-ppm unit for MS/MS.

Progenesis software (Nonlinear Dynamics) was used for relative quantification of peptides, and Proteome Discoverer software (Thermo Fisher Scientific) was used for peptide validation using the fixed-value peptide spectral match (PSM) separator (MASCOT score > 95% identity threshold (middle confidence), MASCOT score > 99% identity threshold (high confidence)) and for post-translational modification (PTM) localization using the phosphorylRS node. Only peptides identified by MASCOT in the Progenesis software and corresponding to peptide groups (same charge, m/z, modifications) validated as middle or highly confident in the Proteome Discoverer software were taken into account. Protein abundances were calculated by summing the abundances of peptides present in the flow-through fraction of IMAC of which no phosphorylated counterpart was retrieved on IMAC. Peptide abundance was determined by the Progenesis software (Nonlinear Dynamics). In the Progenesis software, the calculation of peptide ion abundance is done by summing the areas under the different isotopic peaks belonging to the peptide. The calculation of protein abundance is done by summing abundances of peptides belonging to the protein.

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References


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**PP1 substrate trapping**


