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Mass spectrometry and biochemical analysis of RNA polymerase II: targeting by protein phosphatase-1

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Abstract

Transcription of eukaryotic genes is regulated by phosphorylation of serine residues of heptapeptide repeats of the carboxyterminal domain (CTD) of RNA polymerase II (RNAPII). We previously reported that protein phosphatase-1 (PP1) dephosphorylates RNAPII CTD in vitro and inhibition of nuclear PP1-blocked viral transcription. In this article, we analyzed the targeting of RNAPII by PP1 using biochemical and mass spectrometry analysis of RNAPII-associated regulatory subunits of PP1. Immunoblotting showed that PP1 co-elutes with RNAPII. Mass spectrometry approach showed the presence of U2 snRNP. Co-immunoprecipitation analysis points to NIPP1 and PNUTS as candidate regulatory subunits. Because NIPP1 was previously shown to target PP1 to U2 snRNP, we analyzed the effect of NIPP1 on RNAPII phosphorylation in cultured cells. Expression of mutant NIPP1 promoted RNAPII phosphorylation suggesting that the deregulation of cellular NIPP1/PP1 holoenzyme affects RNAPII phosphorylation and pointing to NIPP1 as a potential regulatory factor in RNAPII-mediated transcription.

Keywords

RNA polymerase II; Protein phosphatase-1; NIPP1; CTD

Background

Transcription of eukaryotic protein-encoding genes is regulated in part by phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II (RNAPII) that contains a tandem array of 26–52 heptapeptide repeats with the consensus sequence Tyr¹-Ser²-Pro³-Thr⁴-Ser⁵-Pro⁶-Ser⁷ [1]. The phosphorylation state of the CTD is the result of combined actions of CTD kinases and phosphatases [2]. Several kinases are involved in the regulation of CTD phosphorylation [3] including cyclin-dependent kinases (CDK), CDK7 and CDK9 which mediate transcriptional elongation as well mRNA transcript maturation. TFIIF-associated CDK7 phosphorylates Ser-5 residues of CTD allowing association of capping enzymes, whereas CDK9 phosphorylates Ser-2 residues of CTD-promoting RNAPII transcription elongation. CTD phosphorylation is also controlled by a number of phosphatases [4–7]. A PP2C-type phosphatase (FCP1) regenerates initiation-competent RNAPII CTD to its non-phosphorylated form [8]. In addition, during transcription elongation, FCP1 can prematurely interrupt transcription by mediating dephosphorylation of RNAPII CTD [9]. A family of small CTD phosphatases (SCP) preferentially catalyzes the dephosphorylation of Ser 5 of CTD [10]. SCP's have been shown to regulate gene transcription; in non-neuronal cells, SCP's are recruited to neuronal genes that contain RE-1 *cis*-elements and silence transcriptional expression of neuronal genes [11]. A component of the yeast cleavage/polyadenylation factor (CPF) complex, Ssu72, was also reported to be a CTD phosphatase with specificity for serine-5 phosphorylated residues [12]. CTD kinases and phosphatases are equally important *trans*-acting factors that regulate transcription, mRNA processing, and RNAPII usage.

Our earlier study showed that protein phosphatase-1 (PP1) dephosphorylated RNAPII CTD in vitro [13], but it remained to be determined whether PP1 influenced RNAPII CTD dephosphorylation in the cells. The three PP1 catalytic subunits, PP1 α , PP1 β/δ , or PP1 γ , bind to a host of different regulatory subunits that determine their localization, activity, and substrate-specificity [14]. Okadaic acid, which inhibits catalytic subunit of PPP family phosphatases including PP1, but not FCP1 [4], has been reported to induce RNAPII phosphorylation in proliferating cultures of HeLa cells [15]. Therefore, it is possible that a PPP-type phosphatase modulates phosphorylation of RNAPII CTD in human cells. Inhibition of the catalytic subunit of PP1 may affect a variety of substrates and be toxic for

the cells. Thus, for a detailed study of PP1's role in RNAPII dephosphorylation, it is important to identify a PP1 regulatory subunit that mediates PP1 interaction with RNAPII complexes. An expanded list of PP1 regulatory subunits has recently been described which include PP1 nuclear regulatory subunits, NIPP1 (nuclear inhibitor of PP1) and PNUTS, and cytoplasmic regulatory subunits, SIPP1 and sds22 [16].

In this study, fractionation of RNAPII complexes by glycerol gradient, followed by immunoprecipitation and mass spectrometry analyses demonstrated that PP1 α co-elutes with RNAPII. Mass spectrometry showed the presence of an U2 snRNP-associated protein. The immunoprecipitation showed association of NIPP1 and PNUTS with RNAPII. Further characterization of the effect of NIPP1 on RNAPII was shown when adeno-associated virus (AAV)-mediated expression of mutant NIPP1, but not the WT NIPP1, induced RNAPII phosphorylation. Inhibition of PP1 catalytic subunit by okadaic acid increased the level of RNAPII phosphorylation in normal cells or cell expressing NIPP1 but not in the cells expressing mutant NIPP1. Lack of RNAPII phosphorylation by okadaic acid with mutant NIPP1 indicates that in the absence of NIPP1 regulation/targeting, PP1 might not be available to associate with CTD resulting in functional inactivity of PP1 for RNAPII dephosphorylation in mNIPP1 expressing cells. Together, our data demonstrate a potential role of NIPP1 as a subunit of PP1 in the regulation of RNAPII dephosphorylation *in vivo*.

Results

PP1 co-elutes with RNAPII

We previously reported that *in vitro* PP1 dephosphorylated RNAPII CTD [13]. It is well known that in the cells RNAPII forms multiple complexes [17]. To determine whether PP1 binds to a complex formed by RNAPII *in vivo*, we analyzed co-elution of PP1 with RNAPII in extracts prepared from human 293T cells and fractionated on glycerol gradients (Fig. 1). PP1 α eluted in high molecular weight fractions along with RNAPII (Fig. 1). PP1 γ eluted mostly in low molecular weight fractions with only a small portion being eluted along with RNAPII (Fig. 1). As a control, we also analyzed co-elution of protein phosphatase 2A (PP2A), which largely migrated in the lower molecular weight fractions and did not co-elute with RNAPII (Fig. 1).

Mass spectrometry analysis of RNAPII-associated proteins

Then, we analyze the RNAPII-associated proteins using mass spectrometry. The 293T cells were lysed and then separated on glycerol gradients (Fig. 2a). Glycerol gradient fractions that contained both RNAPII and PP1 were further purified by affinity chromatography with anti-RNAPII antibodies (Fig. 2a). Proteins were eluted from the column, resolved on 5–15% SDS-PAGE and subjected to trypsin hydrolysis and peptide fingerprinting using FTMS (Fig. 2a, scheme I). Six protein bands that range in MW from 30 to 260 kDa were excised (Fig. 2b, lanes 1–6). The 35 kDa protein was identified as U2 small nuclear ribonucleoprotein auxiliary factor 35 kDa subunit of U2 snRNP splicing complex (Table 1, lane 3) [18]. We further fractionated the proteins eluted from the RNAPII affinity column on microcystin–agarose column with high affinity to PP1 [19] and then subjected to trypsin hydrolysis and peptide fingerprinting using FTMS (Fig. 2a, scheme II). Although we did not detect RNAPII among the Coomassie stained bands, it was detected by immunoblotting in the protein fraction eluted from the microcystin–agarose column suggesting that RNAPII was associated with PP1 (data not shown). Fractions eluted from microcystin column were resolved on 5–15% gradient SDS-PAGE which showed three protein bands (Fig. 2b, lanes 7–9). The bands were excised, and proteins were identified as histone H1.1, H1.3 и β -tubulin TBB2C (Table 1). Thus, we did not detect PP1 regulatory subunits among the RNAPII and PP1-associated proteins. However, identification of U2 snRNAP points to

NIPP1 that was recently shown to be associated with snRNP and that targets PP1 to U2-associated splicing factor SAP155 [20,21]. NIPP1 was also shown to interact with chromatin [22]. Another known PP1 nuclear subunit that interacts with chromatin is PNUTS [23].

Therefore, we analyzed co-precipitation of PNUTS and NIPP1 with RNAPII. As controls, we used SIPP1 and sds22. While NIPP1 and PNUTS co-precipitated with RNAPII, SIPP1 and sds22 did not (Fig. 3). These results indicate that PP1 may be targeted to RNAPII by NIPP1 or PNUTS. Because the mass spectrometry results showed the presence of U2 snRNP that is targeted by NIPP1, we focused on the role of NIPP1 in RNAPII phosphorylation.

Expression of inactive NIPP1 alters phosphorylation of RNAPII CTD

We and others previously showed that RNAPII phosphorylation level was increased in the cells treated with phosphatase inhibitor, okadaic acid, when high micromolar concentrations were used that are inhibitory to PP1 [13,15]. To determine whether NIPP1 can alter the RNAPII phosphorylation, cells were infected with recombinant AAVs that expressed EGFP-fused NIPP1 gene (AAV-NIPP1 wt) or EGFP-fused mutant NIPP1 (AAV-NIPP1 mut) [24]. In the NIPP1 mutant, the PP1 binding sites in both the central and C-terminal domains of NIPP1 are mutated, and it no longer interacts with PP1 [25]. RNAPII CTD was phosphorylated on Ser-2 residues in non-infected cells as well as in the cells infected with the WT vector (Fig. 4a, lanes 3, 5), but the level of phosphorylation was higher in cells infected with the mutant NIPP1 vector (Fig. 4a, lane 5). Because mutated NIPP1 does not bind to PP1, it still has the potential to bind RNAPII complexes, competing with endogenous NIPP1–PP1 complexes, and stoichiometrically reduce the amount of active-PP1 dephosphorylating RNAPII.

Then, we investigated whether changes in RNAPII phosphorylation were due to enzymatic dephosphorylation of RNAPII. We reasoned that if PP1 is no longer involved in the dephosphorylation of RNAPII, then okadaic acid will not prevent dephosphorylation of RNAPII induced by CDK inhibitor flavopiridol. As we previously reported, treatment with 25 μ M flavopiridol induced a rapid dephosphorylation of Ser-2 phosphorylated RNAPII CTD in the cells infected with AAV-NIPP1 or AAV-mutant NIPP1 (AAV-mNIPP1) (Fig. 4b, compare lanes 2, 6, and 10 to the lanes 4, 8, and 12). Treatment of cells with 1 μ M okadaic acid did not have an effect on the overall level of RNAPII phosphorylation (Fig. 4b, lanes 3, 7, and 11). However, pretreatment with okadaic acid before the treatment with flavopiridol reduced dephosphorylation of Ser-2 phosphorylated RNAPII CTD in uninfected and AAV-NIPP1 infected cells (Fig. 4b, lanes 5 and 9). In contrast, okadaic acid did not increase Ser-2 RNAPII phosphorylation in the cells infected with AAV-mNIPP1 (Fig. 4b, compare lane 12 to lane 13). Also, in AAV-mNIPP1 infected cells, basal level RNAPII phosphorylation was increased compared to AAV-NIPP1 infected cells (Fig. 4b, lane 6). These results indicate that expression mNIPP1 makes dephosphorylation of Ser-2 residues of RNAPII CTD insensitive to okadaic acid.

Taken together, these results show that NIPP1 potentially interacts with RNAPII and that expression of NIPP1 mutant, unable to bind and target PP1, induces RNAPII phosphorylation.

Discussion

RNAPII-mediated transcription includes three phases: promoter clearance, productive elongation, and transcriptional termination, which is accompanied by chromatin remodeling, 5' capping, non-coding intervening sequence splicing, and 3' end polyadenylation. Recent studies demonstrate that RNAPII orchestrates these processes [26]. The RNAPII CTD

provides a docking platform for *trans*-acting factors involved in different steps of messenger RNA synthesis [27]. Recruitment of the appropriate factors at different stages of transcription is achieved through alternating patterns of CTD phosphorylation, which create a specific “code” [28]. Dynamic change in CTD phosphorylation allows RNAPII complex to rearrange its component subunits during transcription for sequential enzymatic-action of distinct machineries [27]. During the RNAPII transcription cycle, both the phosphorylation state of the polymerase and the proteins that RNAPII interacts with change dramatically. Throughout promoter recruitment and initiation where RNAPII exists in a hypophosphorylated state, it interacts with general transcription factors, such as TFIID, TFIIB, TFIIF, TFIIIE, TFIIH, and Mediator [29]. In the process of promoter clearance and early transcript elongation, RNAPII becomes hyper-phosphorylated, interactions with initiation-specific factors are severed, and interactions with elongation and splicing complexes are established. Factors that have been shown to preferentially associate with hyper-phosphorylated elongating RNAPII include RNA processing factors and factors thought to play a role in transcript elongation, such as Elongator, Set1, and Set2 [30]. At the end of elongation, RNAPII becomes hypo-phosphorylated and is recycled for a new round of transcription. The reversible phosphorylation of proteins is accomplished by opposing activities of kinases and phosphatases. Several phosphatases (FCP1, SCP) were found to dephosphorylate CTD. FCP1 is associated with RAP74 subunit of TFIIF during elongation and involved in the recycling of RNAPII for a new round of transcription after elongation [31]. Unlike FCP1, SCP preferentially catalyzes the dephosphorylation of Ser 5 within the CTD and is especially active toward RNAPII phosphorylated by TFIIH [32]. Taking into an account the complicity of transcription, it is possible that another phosphatase might be involved in transcription regulation. Indeed, inhibition of PPP-type of phosphatases with okadaic acid increased the level of RNAPII phosphorylation [12]. In contrast to FCP1 and SCP1 monomeric phosphatases, PPP-type phosphatases are dimers that consist of common catalytic subunit and regulatory subunit [33]. PPP regulatory subunits determine location and specificity of a catalytic subunit allowing PPP phosphatases to be involved in multiple cell processes. Therefore, finding a regulatory subunit of PP1 that is involved in RNAPII dephosphorylation is essential. It will allow the future use of genetic tools such as mutational analysis and siRNA silencing to study a role of PP1 in transcription. We previously showed that PP1 associates with RNAPII but the regulatory subunit that targets it to RNAPII was not identified [13].

In this article, we demonstrated that PP1 co-eluted with RNAPII heavy fractions separated by centrifugation in the glycerol gradient. The high salt concentration that we used for whole cell extraction disrupts most DNA–protein interactions but not RNAPII–DNA–RNA complexes that are formed during elongation [30]. Thus, it is possible that PP1 associated with NIPPI or PNUTs may play a role in elongation of transcription. Only small percentage of RNAPII was found to bind to microcystin. It may reflect that PP1 is not bound directly to CTD but rather bind to one or more shuttling elongation complexes. PNUTS was recently found to bind Set1 histone methyltransferase complex and involved in the regulation of chromosome de-condensation at late telophase [34]. We found several histones in the highly purified fractions of RNAPII. They can be tightly bound to DNA in the complex with RNAPII. Thus, PNUTS-PP1 can be involved in the regulation of histone modification during transcription. Future experiments will define if histone modification accompany by change in the CTD phosphorylation.

In this article, we focused on the role of NIPPI in regulation of CTD phosphorylation. NIPPI is required for spliceosome assembly, and it binds U2-associated splicing factor SAP155 and recruits PP1 to dephosphorylate SAP 155 [35]. We identified U2 small nuclear ribonucleoprotein auxiliary factor 35 kDa subunit of U2 snRNP splicing complex by mass spectrometry analysis in RNAPII-purified fraction. This result can indicate that fractionated

RNAPII might contain a spliceosome that may also contain both NIPP and PP1. NIPP1 is a well-described inhibitor of PP1 [25]. However, our results demonstrate that NIPP1 regulates rather than inhibits the activity of PP1 toward dephosphorylation of CTD. Indeed inhibition of catalytic subunit of PP1 by okadaic acid increases level of CTD phosphorylation in normal cells. Overexpression of NIPP1 mutant that lacks PP1 binding motif but is able to bind spliceosome in the absence of PP1 [35] also increases the level of CTD phosphorylation. Addition of okadaic acid together with NIPP1 mutant did not increase CTD phosphorylation further probably because of the absence of bound PP1. NIPP1-associated PP1 does not completely dephosphorylate CTD and, thus, is not likely to be involved in the recycling of RNAPII at the end of transcription elongation as shown for FCP1 [8]. We hypothesized that partially dephosphorylated CTD can provide a temporary stop point or reduce the rate of elongation to couple it with splicing. Future experiments will provide more information whether splicing attachment dynamically regulates CTD phosphorylation.

NIPP1 is also found in a complex with EED and EZH2 two core components of the Polycomb Repressive Complex 2 (PRC2) [36]. PRC2 involved in chromatin remodeling during gene silencing. RNAPII is also located in promoter-proximal regions of silent genes paused in configurations associated with hypophosphorylated form [37]. It is likely that PP1 bound to NIPP1 plays a yet unrecognized role in the silencing of cellular genes similar to the recently described SCP1 function in silencing of neuronal genes outside of the neuronal tissue [38]. Indeed selective inhibition of nuclear PP1 in forebrain neurons in transgenic mice is shown to induce several histone modifications including phosphorylation and altered gene transcription [39]. In our experiments, we did not find components of Polycomb Repressive complex in RNAPII fraction associated with PP1. One of the possible explanations that condition of nuclear extract preparation disrupted this complex that does not contained RNA.

We did not find directly regulatory subunits of PP1 by mass spectrometry analysis. This may be due to the limitations of the MASCOT search. When we used Findmod program (<http://expasy.org/tools/findmod/>) that match peptides to known proteins, we found 20 peptides matching to the large subunits of RNAPII; 6 peptides matching to PP1 α ; and 3 peptides matching to NIPP1 (data not shown). Thus, more detailed investigation with the use more advances MS/MS analysis or better search tools may provide additional information about RNAPII-associated PP1. Future study of the proteins associated with NIPP1-PP1 and RNAPII will allow better understanding of the role of CTD phosphorylation in transcription.

Methods

Materials

HEK 293T cells and 84–31 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 50 μ g/ml streptomycin/penicillin (all from Invitrogen). Rabbit anti-PP1 γ and anti-PP1 α antibodies were from Calbiochem (Gibbstown, NJ). Monoclonal antibodies specific to the unphosphorylated form of CTD (8WG16) or CTD phosphorylated on Serine 2 (H5) were purchased from BabCo (Richmond, CA). Okadaic acid was purchased from Sigma (Atlanta, GA). Anti-NIPP1, anti-PNUTS, and anti-sds22 antibodies described in [40] and anti-SIPP1 described in [41] were kindly provided by Monique Beullens and Mathieu Bollen (Catholic University of Leuven, Belgium). Antibodies against A-subunit of PP2A were from Upstate (Lake Placid, NY). Flavopiridol ((-)-*cis*-2-(2-chlorophenyl)-5,7-dihydroxy-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4H-1-benzopyran-4-one hydrochloride) was a gift from Dr. Kashanchi (The George Washington University, Washington DC). The expression vectors for EGFP-fused central domain of NIPP1, NIPP1-(143–224), or NIPP1-(143–224) V201AF203A

(RATA mutant) described in [25] were kindly provided by Monique Beullens and Mathieu Bollen (Catholic University of Leuven, Belgium). NIPP1 mutated within its RVXF motif has low binding affinity for PP1 [25].

Recombinant adeno-associated virus

Recombinant AAV vectors containing either the EGFP-fused NIPP1 gene under the control of the CMV promoter (AAV-NIPP1) or mutant EGFP-fused NIPP1 K193-197A/V201A/F203A/Y335D (AAV-NIPP1_{PARATA}) were produced as described [24]. In brief, the *cis* plasmid (which contains the gene of interest with AAV ITRs), the *trans* plasmid (with the AAV *rep* and *cap* gene), and a helper plasmid (pFΔ6, which contains an essential region from the Ad genome) were co-transfected into 293 cells at a ratio of 1:1:2 by calcium phosphate precipitation in an Ad-free system. The cells were harvested 96 h later. The AAV was purified through two rounds of CsCl density gradient centrifugation, desalted by dialysis at 4°C against PBS, aliquoted, and kept at 4°C. The titer was determined by quantitative dot blot hybridization. The purity of the AAV vector preparation was addressed by colloidal brilliant blue G staining (Sigma, St. Louis, MO) and Western blotting of viral proteins separated by SDS-PAGE.

Western blot analysis of RNAPII

Confluent 84–31 cells were lysed in SDS Sample Buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 0.2 M β-Mercaptoethanol, 0.0025% bromophenol blue). Protein lysates were separated by SDS-PAGE and then electrophoretically transferred to PVDF (Millipore) membrane at a constant 150 mA (~15 V) overnight at 4°C. After wetting the membrane in pure methanol, the membrane was blocked in 5% (w/v) non-fat milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) buffer for an half-hour at room temperature, washed twice in fresh changes of PBS-T with agitation, followed by once in PBS-T for 5 min. RNAPII was detected with primary antibodies against non-phosphorylated (8WG16, 1:2000) or Ser-2 phosphorylated CTD (H5, 1:500 dilution) for 1 h at room temperature. Membranes were washed as above, and then probed with anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:2000), for 30 min at room temperature, followed by washing. Chemiluminescence detection of proteins was conducted using the ECL Western Blot Analysis System™ detection reagents (Amersham Biosciences, England) exposed to Kodak Biomax film™ (Kodak).

Immunoprecipitation of RNAPII

HeLa or 84–31 cells were lysed in whole cell lysis buffer (50 mM Tris–HCl, pH 7.5, 1% NP-40, 0.1% SDS, 0.25 M NaCl). Whole cell extract (50 µg) was supplemented with 2 µg of anti-RNAPII-CTDa monoclonal antisera coupled to protein-A/G agarose in TNN Buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40). Immunoprecipitation was carried out for 2 h at 4°C. Antigenic complement coupled beads were washed three times with 0.5 ml of TNN buffer and once with 50 mM Tris–HCl, (pH 8.0). Proteins were resolved on 4–12% SDS-PAGE, transferred to PVDF, and Western blotted as described above. Co-immunoprecipitation assays were carried out using monoclonal mouse antibodies specific for RNAPII antigen and then western blot analyzed with rabbit or goat antibodies specific for NIPP1 (1:2000), SIPP1 (1:1500), sds22 (1:1000), or PNUTS (1:1500) regulatory proteins as described above.

Phosphorylation of RNAPII CTD in HeLa nuclear extract

RNAPII was phosphorylated in transcription reactions (20 µl) containing 50 µg of HeLa nuclear extract, 0.5 mM ATP, CTP, UTP and GTP, and 0.2 µg of JK2 [42] linearized template in buffer C (20 mM HEPES, pH 7.9, 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM

EDTA, 2 mM DTT, and 10% glycerol). Transcription was carried out for 30 min at 30°C. The reaction was terminated by addition of 7 mM EDTA.

Analysis of RNAPII phosphorylation in cultured cells

84–31 cells and cells infected with either AAV-NIPPI1 or AAV-mutNIPPI1 were cultured in 24-well plates with DMEM containing 10% FBS and antibiotics. At 75% confluency, cell cultures were treated with okadaic acid (0.01 or 1 μ M) for 30 min. After treatment, cells were washed in PBS and lysed with SDS-loading buffer. Lysates were adjusted to equal protein concentration and resolved on 4% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and analyzed by immunoblotting with 8WG16, or H5 monoclonal antibodies against non-phosphorylated or Ser-2 phosphorylated RNAPII CTD.

Fractionation of cellular lysates on glycerol gradient

293T cells were grown in 100-mm plates, then lysed with 0.5 ml of whole cell lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1% NP-40, 0.1% SDS) supplemented with protease inhibitors cocktail (Sigma). Cell lysates were clarified by centrifugation for 30 min at 10,000g and loaded on top of 10–30% glycerol (9 ml) gradient. Glycerol gradient buffer contained 20 mM HEPES-KOH, pH 7.9, 150 mM KCl, 0.2 mM EDTA. The gradient was spun in a Sorvall SW41 Ti rotor using XL-90 Ultracentrifuge (Beckman) at 38,000 rpm for 18 h. Twenty fractions (0.5 ml) were collected through a needle inserted to the bottom of the tube using fraction recovery system (Backman), and the proteins in each fraction were precipitated by 70% trichloroacetic acid and analyzed by immunoblotting for indicated proteins.

Affinity column with anti-RNAPII antibodies

About 2 ml of NHS-activated Sepharose (GE Healthcare 17-0906-01) was suspended in isopropanol and washed twice with 20 ml of 1 M HCl at 4 °C. After each wash, the resin was collected by centrifugation. The Sepharose was then incubated with 200 μ g of monoclonal anti-RNAPII antibodies in PBS overnight at 4°C. Then, the coupling reaction was blocked by the addition of 0.1 M Tris-HCl (10 ml, pH 8.5) for 2 h at 4°C, washed twice with 0.1 M Tris-HCl (pH 8.5), and precipitated by centrifugation at 500 $\times$ g. The resin was then washed five times with 0.1 M Na-Acetate (pH 4.4), transferred to a mini-column, and equilibrated with TBS buffer (20 mM Tris-HCl pH 7.5, 137 mM NaCl). Proteins were suspended in TBS and passed through the column. The bound proteins were eluted with 0.1 M Na-Acetate (pH 4.4).

Microcystin-agarose chromatography

Microcystin-agarose washed with 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 0.02% Tween-20. Proteins eluted from the RNAPII affinity column were incubated with microcystin-agarose overnight at 4°C on a rocker. Then, the agarose was washed with 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 0.02% Tween-20 and with 0.1 M Tris-HCl, (pH 6.8). Then, the agarose was suspended in the SDS-loading buffer and boiled for 5 min, and the supernatant was loaded on SDS-PAGE.

Preparation of samples for mass spectrometry analysis

The SDS-PAGE gel was washed in water three times for 10 min and then stained with colloidal Coomassie Blue overnight. Protein bands were excised from the gel with a razor, and gel pieces were transferred to eppendorf tubes and washed twice with 50% Acetonitrile containing 0.1% TFA. Coomassie stain was removed by incubation of the gel in 0.2 ml 100 mM NH_4HCO_3 dissolved in 50% Acetonitrile. Then, the gel was dehydrated in 100% Acetonitrile and dried in a SpeedVac. The gel was rehydrated in 40 mM NH_4HCO_3 , 10%

acetonitrile containing 20 µg/ml sequence grade trypsin and then incubated overnight at 37°C, and the supernatant was collected. The gels were re-extracted with 50% acetonitrile 5% TFA for 1 h. The extracts were combined and dried in SpeedVac. ZipTips were prewashed with 100% Acetonitrile followed by a wash with 0.1% TFA. Extracted peptides were dissolved in 0.1% TFA, loaded into ZipTip, washed with 0.1% TFA, and eluted with 70% acetonitrile, 0.1% TFA.

Mass spectrometry analysis

About 0.4 µl of eluted peptides was mixed on the target plate with 0.4 µl 2,5-dihydroxybenzoic acid (DHB) (Aldrich) diluted to 20 mg/ml in 50% Acetonitrile and 0.1% TFA and then dried under vacuum. Mass spectrometric analysis was conducted on Fourier Transform (Ion Cyclotron Resonance) Mass Spectrometer (Varian 902-MS) equipped with MALDI and 9.4 T magnet (FTMS). Samples were irradiated by series of five impulses at 355 nm pulses from a third harmonic of neodymium-doped yttrium aluminum garnet (Nd:YAG) laser. The laser power was set to the minimum level necessary to generate a reasonable signal. Signal from five shots was recorded. A ProteoMass Peptide MALDI-MS Calibration Kit (Sigma) was used for external calibration. For internal mass calibration, residual trypsin peaks (842.50940 and 2211.10400 Da) were used. Proteins were identified using Mascot (<http://www.matrixscience.com>).

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Fig. 1. Co-purification of PP1 α catalytic subunit with RNAPII. Whole cell extracts were prepared from 293T cells and separated on glycerol gradients by ultra centrifugation. The gradients were fractionated and analyzed by Western blotting for RNAPII, PP1 α , PP1 γ , and the A-subunits of PP2A

Fig. 2. Mass spectrometric analysis of RNAPII-associated proteins. RNAPII-associated proteins were separated by centrifugation in glycerol gradient, resolved on 5–15% SDS-PAGE and stained with Colloidal Coomassie. Proteins bands indicated were excised and processed for mass spectrometric analysis. **a** Schematic representation of the experiment. **b** Photograph of Coomassie stained gels generated in panel A. I and II experiments from schemes I and II from panel A

Fig. 3.

NIPPI1 and PNUTS co-precipitate with RNAPII. RNAPII was immunoprecipitated from HeLa cell extract with 8WG16 monoclonal antibodies. Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE and immunoblotted with anti-NIPPI1 (*lane 2*), anti-PNUTS (*lane 4*), anti-SIPP1 (*lane 6*), and anti-sds22 (*lane 8*) antibodies. Lanes 1, 3, 5, and 7—inputs. Asterisk indicates the position of heavy chain of IgG

Fig. 4.

Expression of mutant NIPP1 alters phosphorylation RNAPII CTD. **a** Expression of mutant NIPP1 induces phosphorylation of RNAPII CTD. 84-31 cells were infected with recombinant AAVs expressing EGFP-fused NIPP1 (AAV-NIPP1 wt) (lane 4) or EGFP-fused mutant NIPP1-K193-197A/V201A/F203A/Y335D (AAV-NIPP1 mut) (lane 5). Lane 1 HeLa nuclear extract. Lane 2 RNAPII phosphorylated in HeLa nuclear extract under in vitro transcription conditions. Lane 3 control uninfected 84-31 cells. Whole cell lysates were prepared, resolved on 5% SDS-PAGE and analyzed by Western blot with serine 2 phospho-epitope-specific antibodies (*upper pane*) and with antibodies against non-phosphorylated RNAPII (*lower pane*). **b** Expression of mutant NIPP1 makes dephosphorylation of RNAPII CTD insensitive to okadaic acid. 84-31 cells, uninfected (84-31 cells, lanes 2 to 5); infected with AAV-NIPP1 (84-31 cells with NIPP1, lanes 6 to 9); or AAV-NIPP1 mutant (84-31 cells with mut NIPP1, lanes 10 to 13) were treated where indicated with 25 μ M flavopiridol for 60 min to promote RNAPII dephosphorylation. Where indicated, the cells were also treated for 30 min with 1 μ M okadaic acid before the treatment with flavopiridol to block PPP phosphatases. Whole cell lysates were prepared, resolved on 5% SDS-PAGE, and analyzed by Western blot with serine 2 phospho-epitope-specific antibodies. Lane 1 HeLa nuclear extract subjected to in vitro transcription conditions to phosphorylate RNAPII. The hyperphosphorylated (Ilo) form of RNAPII is indicated

Table 1
Determination of RNAPII-associated proteins by mass spectrometry and MASCOT search

# of stained band	Band experimental molecular weight, kDa	MS prediction protein name	Accession number	GenPept	Predicted protein molecular weight, kDa	Expect	Number of mass values matched	Sequence coverage, %
1	280	Spectrin beta chain, brain 3	NP_066022		289	0.021	31	15
2	65	Protein 4.1	NP_001159477		97	0.00089	26	26
3	55	U2 small nuclear ribonucleoprotein auxiliary factor	NP_001012496		58	0.00044	34	51
4	45	Actin, cytoplasmic 1	NP_001092		42	0.005	10	31
5	35	Annexin A5	NP_001145		36	0.0033	11	42
6	30	1-aminocyclopropane-1-carboxylate synthase-like protein 1	NP_115981		57	0.022	12	27
7	50	Tubulin beta chain	NP_821133		50	1e-06	15	37
8	45	Histone H1.1	NP_005316		22	0.023	18	72
9	25	Histone H1.3	NP_005311		22	9.5e-05	18	45