RRP1B Targets PP1 to Mammalian Cell Nucleoli and Is Associated with Pre-60S Ribosomal Subunits

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A pool of protein phosphatase 1 (PP1) accumulates within nucleoli and accounts for a large fraction of the serine/threonine protein phosphatase activity in this subnuclear structure. Using a combination of fluorescence imaging with quantitative proteomics, we mapped the subnuclear localization of the three mammalian PP1 isoforms stably expressed as GFP-fusions in live cells and identified RRP1B as a novel nucleolar targeting subunit that shows a specificity for PP1β and PP1γ. RRP1B, one of two mammalian orthologues of the yeast Rrp1p protein, shows an RNAse-dependent localization to the granular component of the nucleolus and distributes in a similar manner throughout the cell cycle to proteins involved in later steps of rRNA processing. Quantitative proteomic analysis of complexes containing both RRP1B and PP1γ revealed enrichment of an overlapping subset of large (60S) ribosomal subunit proteins and pre-60S nonribosomal proteins involved in mid-late processing. Targeting of PP1 to this complex by RRP1B in mammalian cells is likely to contribute to modulation of ribosome biogenesis by mechanisms involving reversible phosphorylation events, thus playing a role in the rapid transduction of cellular signals that call for regulation of ribosome production in response to cellular stress and/or changes in growth conditions.

INTRODUCTION

The primary role of the nucleolus, a nonmembrane-bound organelle that forms around tandem repeats of rDNA in the nucleus, is to ensure that the cell receives the essential supply of ribosomes required for protein synthesis (for review see Boisvert et al., 2007). Because it must respond to dynamic changes in cell growth rate and metabolic activity with either an increase or decrease in ribosome subunit biogenesis, tight control of nucleolar pathways of rDNA transcription and ribosome subunit processing and export is critical. Several key cellular kinases and phosphatases have been linked to regulation of nucleolar events throughout the cell cycle, and our work has shown that the serine/threonine protein phosphatase 1 (PP1) accounts for ∼80% of Ser/Thr phosphatase activity within this structure (Trinkle-Mulcahy et al., 2003).

The intracellular localization and substrate specificity of the core catalytic subunit of PP1 is regulated through its association with a spectrum of interacting proteins, termed targeting subunits (for review see Cohen, 2002). Most of these targeting subunits contain conserved motifs that mediate direct binding to PP1, including the “RVxF” motif with its consensus Arg/Lys-Val/Ile-Xaa-Phe/Trp (Egloff et al., 1997; Wakula et al., 2003). Over the years biochemical, bioinformatic, and proteomic approaches have been used to identify and characterize a wide range of PP1 targeting subunits (Tran et al., 2004; Meiselbach et al., 2006; Trinkle-Mulcahy et al., 2006; Roadcap et al., 2007; Moorhead et al., 2008; Hendrickx et al., 2009), however the current list still cannot account for the large number of regulatory pathways in which PP1 is known to play a critical role.

Of the three closely-related mammalian isoforms, PP1α, PP1β, and PP1γ (Barker et al., 1993; Shima et al., 1993; Barker et al., 1994), only the β and γ isoforms show significant accumulations within nucleoli (Trinkle-Mulcahy et al., 2001; Trinkle-Mulcahy et al., 2003; Trinkle-Mulcahy et al., 2006; Lesage et al., 2005; Andreassen et al., 1998). This difference has been attributed to a specific N-terminal Arginine residue (Arg19 in PP1β and Arg20 in PP1γ) that is not present in PP1α (Lesage et al., 2005). Having validated the use of FP-PP1 fusion proteins as markers for endogenous pools of PP1 (Trinkle-Mulcahy et al., 2001), we went on to demonstrate that isoform-specific binding partners detected via affinity purification of the respective tagged proteins reflect the
Plasmids and Antibodies

complexes. that is associated with pre-60S ribosomal subunit processing

the granular component of mammalian cell nucleoli

remain associated throughout this process.

prophase is initiated by inhibition of transcription, and re-

assemblies (Chamousseau et al., 2010). As presented here, nucleolar interactor screens of GFP-PP1 define a wide range of multiprotein complexes to which the phosphatase is targeted, including complexes involved in ribosome subunit biogenesis.

The pathway of ribosome subunit biogenesis is initiated in the nucleolus by RNA Pol I–mediated transcription of pre-rRNAs (Russell and Zomerdijk, 2005). This transcript is further processed into 18S transcripts (which form the 40S subunit). The nucleolar proteins assemble on these complexes (RP1s with 60S, RP2s with 4OS), where they are joined by a large number of nonribosomal processing proteins. In yeast, >300 proteins, many of which show transient associations, have been characterized or predicted to play roles in the maturation and export of pre-ribosomal particles (Nissan et al., 2002). Affinity purification-based analysis of mutant strains involved in ribosome subunit biogenesis.

Materials and Methods

Plasmids and Antibodies

All FP-PP1 constructs were described previously (Trinkle-Mulcahy et al., 2001; Trinkle-Mulcahy et al., 2003). PP1b-EGFP was prepared by subcloning the PP1b cDNA into the EGF-N3 vector. The EYFP-NLS plasmid (nuclear localization signal cloned into EYFP-C1), which accumulates in nucleoli in addition to its cytoplasmic and nuclear localization, was a generous gift from De Archa Fox (Western Australia Institute for Medical Research, Australia). RRP1b was cloned from an expressed sequence tag using oligonucleotide primers and inserted into EGF-P/myc/ET vectors. The Vc and Ph residues of the putative PP1 binding motif (residues 684 and 686) were changed to Ala using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Noll, RLP2, and RPS23 were cloned and inserted into the EGF-P-C1 vector, while EGF/YFP-tagged RPA39, Fibrillarin, PWP1, Gar1, B23, Pescador, and RPL27 were obtained as previously described (Leung et al., 2004). YFP-RNR3 and GFP-NP1/Nopt2 were generous gifts from Drs. Joost Zomerdijk (University of Dundee, UK) and Daniela Hernandez-Verdun (Institut Jacques Monod, France). Recombinant His-tagged RRP1b was expressed in bacteria, purified using Ni2+–NTA beads, and injected into rabbits for the generation of the polyclonal antibodies used in this study.

Far Western Blotting

Recombinant purified wild-type and KATA mutant RRP1b (200 ng each) were electrophoresed and transferred to nitrocellulose membrane, along with control lanes containing 10 µg of total protein from rat nuclear extracts. The membranes were incubated with 10% milk to block nonspecific binding sites and then overlaid with either DIG-labeled recombinant PP1α or PP1γ. Anti-DIG-HRP antibodies (Pierce; Rockford, IL) were used to detect the binding profiles of the PP1 isoforms.

Cell Culture and Transfection Assays

HeLa and 2OS cells were obtained from ATCC (Manassas, VA). Cells were grown in Dulbecco’s modified Eagles’ medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and streptomycin (Wisent). For immuno-

fluorescence assays, cells were grown on cover slips coated with Vaps (Polysciences). The microscope was controlled by SoftWoRx acquisition and deconvolution software (Applied Precision). DNA was stained by incubating the cells for 30 min in medium containing 0.25 µg/ml Hoechst No 33422 (Sigma-Aldrich, St. Louis, MO). For FRAP experiments, cells was imaged before photobleaching, a region of interest was then bleached to ~50% of its original intensity using the 488-nm laser, and a rapid series of images was acquired after the photobleach-

ing period. Recovery curves were plotted and the mobile fraction and half time of recovery were determined using SoftWoRx and Excel (Microsoft, Redmond, WA).

Live Cell Imaging

Time-lapse imaging and FRAP experiments were carried out as described previously (Trinkle-Mulcahy et al., 2006; Trinkle-Mulcahy et al., 2007), using a wide-field fluorescence microscope (DeltaVision CORE; Applied Precision, Issaquah, WA) equipped with a three-dimensional motorized stage, temperature- and gas-controlled environmental chamber, and 488-nm diode laser (for photobleaching EGFP). Images were collected using a 60 × NA 1.4 Plan-Apochromat objective and recorded with a CoolSNAP coupled-charge-device (CCD) camera (Roper Scientific, Trenton, NJ). DIC imaging was obtained with the appropriate prism insert. The microscope was controlled by SoftWoRx acquisition and deconvolution software (Applied Precision). DNA was stained by incubating the cells for 30 min in medium containing 0.25 µg/ml Hoechst No 33422 (Sigma-Aldrich, St. Louis, MO). For FRAP experiments, cells was imaged before photobleaching, a region of interest was then bleached to ~50% of its original intensity using the 488-nm laser, and a rapid series of images was acquired after the photobleaching period. Recovery curves were plotted and the mobile fraction and half time of recovery were determined using SoftWoRx and Excel (Microsoft, Redmond, WA).

RNAse and DNase Treatments

Cells were grown on glass coverslips, rinsed once with PBS and once with ASE buffer (20 mM Tris pH 7.5, 5 mM MgCl2, 0.5 mM EGTA), and permeabilized by incubating for 5 min at room temperature in ASE buffer plus 0.1% Triton X-100. Cells were then treated for 20 min at room temperature with either PBS (mock treatment), RNAse (100 µg/ml; Worthington, Lakewood, NJ), or DNase (1000 U/ml; Worthington). After treatment the cells were rinsed with PBS and fixed with 3.7% PFA in CSK buffer for 5 min. After 10 min permeabilization with 1% Triton X-100 in PBS, cells were stained sequentially with the rabbit polyclonal anti-RRP1b primary antibody, DyLight488 anti-rabbit secondary antibody (Pierce), Hoechst 33342 (2.5 µg/ml for 2 min), and Pyronin Y (33 mM for 5 s). Coverslips were mounted on glass slides using FluorSave mounting media (Merck, Whitehouse Station, NJ).

Preparation of Whole Cell Lysates and Immunoblotting

Cells were washed twice with ice-cold PBS and lysed in 0.5 ml of ice-cold 50 mM Tris-HCl pH 7.5; 0.5 M NaCl; 1% (vol/vol) Nonidet P-40; 1% (wt/vol) sodium deoxycholate; 0.1% (wt/vol) sodium dodecylsulfate. 2 ml of 1× LDS buffer, and protease inhibi-

tor cocktail (Roche, Indianapolis, IN). The lysate was passed through a QasheRedder column (Qaigen) to shear DNA and cleared by centrifugation at 34,000 g for 10 min at 4°C. Lysates were separated on 4–12% Novex Nu-PAGE Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes for immunoblotting. Primary antibodies used were anti-GFP mouse monoclonal (Roche), isoform-specific goat anti-PP1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Noll (ProteinTech Eu-
of UV absorbance was obtained upon fractionation of the extracts of a sucrose gradient, with continuous monitoring at 254 nm using a UA-6 UV detector (ISCO, Lincoln, NE) and UNICORN 5.01 software (GE Healthcare). One-milliliter fractions were collected, with 0.9 ml of each used for protein extraction by TCA precipitation, and the remaining 0.1 ml for RNA extraction using Trizol (Invitrogen).

**Reverse Transcription and 28S PCR**

cDNA for each RNA sample was synthesized using the AMV Reverse Transcriptase kit (Promega, Madison, WI), as per the manufacturer’s instructions. PCR was carried out using GoTag Flexi DNA polymerase kit (Promega) and a MasterCycler ProPCR machine (Eppendorf, Hamburg, Germany). The following primers were used to amplify 28S rRNA: GTCACCACACTAATAGGGAGAAC for 28S rRNA FWD and GAGTTCTGACTTAGAGGGCCTT for 28S rRNA RREV (Rybak, 2004). For each sample, 10 μl of PCR product was separated on a 1% agarose gel and stained with SYBR Safe DNA gel stain (Invitrogen). Gels were imaged using a Fuji LAS 4000 Mini Chemiluminescent Imager.

**In Vivo Transcription Assays**

U2OS cells were grown on coverslips, incubated at 37°C for 20–40 min with 1 ml of 5-fluorouridine (Sigma) and fixed for 5 min with PFA-CSK as described above. After permeabilization with 0.5% Triton X100 for 10 min, the incorporated FD was detected using anti-BrdU (Sigma) primary and Dylight 549 anti-mouse (Thermo Scientific) secondary antibodies. All coverslips were stained with Hoechst 33342 and mounted in FluorSave mounting media (Calbiochem) for imaging.

**Northern Blots**

Total cellular RNA was extracted from HeLa cells using the RNasy Mini Kit (Qiagen). U2OS was separated for 3 h at 110 Volts (NorthernMax Kit, Ambion), transferred to BrightStar-Plus Positively Charged Nylon Membrane (Ambion, Austin, TX) for 2 h using a TurboBlotter (Whatman), and biotinylated probes detected by streptavidin reagent (BrightStar BioDetect Kit, Ambion). BrightStar Biotinylated RNA Millenium Markers (Ambion) were included on each blot. The sequences of the 5′ biotinylated probes (Dharmacon) used to detect pre-rRNA species are as follows: human pre-rRNA probe hITS1 (1076-1091) 5′ biotin-AGGTCGATTTGGCGAG and human pre-rRNA probe hITS1 (869-884) 5′ biotin-GACACCCACCCACGAG.

**RESULTS**

**RRP1B Is a Nucleolar PP1 Targeting Subunit**

As our previous work indicated the importance of PP1 in nucleolar processes (Trinkle-Mulcahy et al., 2003), we set out to define the nucleolar PP1 interactome using a quantitative proteomics-based approach. HeLa and U2OS cell lines stably expressing the three FP-tagged PP1 isoforms at low levels were established and characterized (Trinkle-Mulcahy et al., 2001; Trinkle-Mulcahy et al., 2003; Andreassen et al., 1998). Fluorescence imaging of both fixed and live cells confirmed that the fusion proteins maintain the distinct subcellular localization patterns of their endogenous counterparts, including the accumulation of PP1β-GFP and GFP-PP1γ in nucleoli (Figure 1, B and C). PP1α distributes equally throughout the nucleoplasm and nucleoli (Figure 1A). Nucleoli are readily purified from these cell lines in large quantities and with high purity, as shown here for HeLa EYFP-PP1α (Figure 1D, inset), and the nucleolar pool of GFP-PP1 is retained throughout the purification protocol.

We initially immunoprecipitated GFP-PP1γ and nucleolar-targeted YFP (as a negative control) from nucleolar extracts derived from HeLaEGFP-PP1γ and HeLaEYFP-NLS cells (according to Trinkle-Mulcahy et al., 2008). We quantified all of the proteins identified and found KIAA0179/RRP1B to be the most abundant putative PP1 interaction partner (Figure 1E). After our optimization of nucleolar protein extraction (Chamousset et al., 2010), we repeated this experiment with similar results (Figure 6D) and also identified RRP1B as an interaction partner for nucleolar GFP-tagged PP1β (data not shown). In addition, RRP1B was identified as a PP1 interactors in HeLa nuclear extracts using a peptide displacement chromatography method (Moorehead et al., 2008).
RRP1B contains a “Nucleolar Protein of 52 kDa” (NOP52) homology domain and the canonical “RVxF” motif (Figure 1E) found in most PP1 targeting subunits (Wakula et al., 2003). In immunoprecipitation experiments, endogenous PP1γ and PP1β, but not PP1α, copurified specifically with GFP-RRP1B (Figure 1F). This interaction was disrupted when the hydrophobic Val and Phe residues in the RVxF motif were mutated to Ala (RRP1B-KATA, Figure 1G). The specificity of binding to PP1γ over PP1α and that the KATA mutation greatly weaken its ability to bind PP1. Equal amounts of purified recombinant DIG-labeled PP1α and PP1γ were overlaid on 200 ng of purified recombinant wild type and KATA mutant RRP1B. The control lane (10 μg rat liver nuclear extract) illustrates that DIG-PP1α binds other target proteins with high affinity.

Nucleolar RRP1B Localization Is RNA-Dependent
The RRP1B antibody signal shows significant colocalization with the Pyronin Y–labeled RNA signal in nucleoli in both U2OS (Figure 2C) and HeLa (Figure 2E) cells. Treatment of cells with RNAse results in a near total loss of the RNA and nucleolar RRP1B antibody signals in both cell lines (Figure 2D and F). Because the cells are lightly permeabilized to permit access of the RNAse, any RRP1B protein displaced by digestion of RNA would be lost. In contrast, neither mock treatment nor DNAse treatment affects the localization of endogenous RRP1B (Supplemental Figure 1).

GFP-RRP1B Shows a Similar Localization to the Endogenous Protein and Can Recruit Excess PP1 to the Nucleolus when Overexpressed
We established a U2OS cell line stably expressing GFP-RRP1B and compared localization of the fusion protein with that of the endogenous protein. Both are predominantly nucleolar (Figure 2, A and B, arrows). Additional accumulations were observed in the perichromatin region in metaphase cells (Figure 2A, arrowhead) and in cytoplasmic foci in late telophase cells (Figure 2B, hashed arrows).
nucleolar (Figure 3A, arrow) in interphase cells and also show a similar distribution throughout the cell cycle, first associating with the perichromatin region during metaphase and early anaphase (Figure 3B, arrowheads) and later ap-
pearing in small foci during telophase (Figure 3B, hashed arrow), before reaccumulation in nucleoli during G2.

Previous studies have shown that overexpression of a PP1 targeting subunit can cause redistribution of the PP1 catalytic subunit to the perichromatin region during metaphase (Trinkle-Mulcahy et al., 2001; Trinkle-Mulcahy et al., 2006). Similarly, overexpression of mCh-RRP1B in HeLa cells also shows similar colocalization in nucleoli (arrows) of RRP1B (green) and Pyronin Y-stained RNA (red) and also demonstrate the sensitivity of RRP1B to RNAse treatment (F). Scale bars are 15 μm.

**Figure 2.** Localization of endogenous RRP1B is predominantly nucleolar and sensitive to RNAse treatment. (A) In interphase U2OS cells, endogenous RRP1B (red) is predominantly nucleolar (arrow). The metaphase cell in this field shows perichromatin accumulations of RRP1B (arrowhead). (B) The telophase cell in this field shows that RRP1B is found initially in bright foci that likely represent pre-
nucleolar bodies (PNBs; hashed arrows) at the end of mitosis, and later in mature nucleoli (arrows). (C) Endogenous RRP1B stained with anti-RRP1B antibodies (green) in U2OS cells colocalizes with the nucleolar RNA signal (arrow) visualized by Pyronin Y staining (red). (D) Treatment of live cells with RNAse before fixation results in a near complete loss of both the RNA and anti-RRP1B, but not the DNA signal. (E) HeLa cells show a similar colocalization in nucleoli (arrows) of RRP1B (green) and Pyronin Y-stained RNA (red) and also demonstrate the sensitivity of RRP1B to RNAse treatment (F). Scale bars are 15 μm.

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**Figure 3.** GFP-tagged RRP1B shows a similar localization to the endogenous protein, and overexpression of exogenous RRP1B alters nucleolar levels of PP1. (A) GFP-RRP1B stably expressed in U2OS cells shows a similar nucleolar accumulation (arrow) to the endogenous protein, as shown here by a fluorescent signal superimposed on a differential interference contrast (DIC) image of the cells. DNA stained with Hoechst 33342 is shown in blue. (B) The fusion protein GFP-RRP1B also shows similar perichromatin accumulations during metaphase and anaphase (B, arrowheads) and likely prernucleo-
lar body (PNB) accumulations in telophase (hashed arrow). DNA stained with Hoechst 33342 is shown in red. (C) Overexpression of mCherry-RRP1B (red) in U2OS/GFP-PP1γ cells leads to an increased level of GFP-PP1γ (green) in the nucleolus (arrow) compared with the nucleoplasm. (D) Overexpression of the mCherry-RRP1B KATA mutant in U2OS/GFP-PP1γ cells leads to a decreased level of GFP-PP1γ (green) in the nucleolus (arrow) relative to the nucleoplasm. (E) High levels of mCherry-RRP1B KATA overexpression induce changes in nucleolar morphology, specifically a “rounding up” (arrow), and these nucleoli show reduced levels of 5-fluorouridine incorporation compared with nucleoli in nontransfected cells (hashed arrows). All experiments were repeated three times in two different cell lines. Scale bars are 15 μm.

**RRP1B Is a Predominantly GC-Associated Nucleolar Protein**

To determine the predominant subnucleolar localization of RRP1B, YFP/GFP-tagged protein markers for the fibrillar

PP1β and PP1γ. Conversely, overexpression of the nonPP1 binding GFP-RRP1B KATA mutant decreases the nucleolar pool of GFP-PP1γ (Figure 3D). High levels of GFP-

RRP1B KATA expression also induced a “rounding up” of nucleoli (Figure 3E, arrow). This “rounding up” is accompanied by a clear reduction of nucleolar 5-fluorouridine incorporation (Figure 3E, arrow) when compared with cells expressing little or no mutant protein (Figure 3E, hashed arrow).
centr (FC), dense fibrillar component (DFC), and granular component (GC), respectively, were coexpressed with mCherry-RRP1B (Figure 4A). The RRP1B signal is distinct from that of the FC markers RPA39 and RRN3 and the DFC markers Filibrin and Gar1 but colocalizes with the GC markers B23 and Pescadillo.

Time-lapse triple-wavelength imaging of mCh-RRP1B, GFP-B23 and Hoechst 33342-stained DNA revealed that these two granular component proteins also exhibit similar localization patterns throughout mitosis (Figure 4B). Specifically, when nucleoli break down at the onset of prometaphase, both proteins become predominantly diffuse, with additional accumulations observed in the perichromatin region of the condensed chromosomes (Figure 4B, arrowhead). During late telophase, accumulations are observed in prenucleolar bodies (PNBs; Figure 4B, arrows). The contents of these PNBs later appear in newly-formed nucleoli (Figure 4B, hashed arrows). Although many of the PNBs disappear over time as their contents transfer to nucleoli, fusion of small bodies into larger nucleoli over time was also observed (Figure 4C; hashed arrows).

**RRP1B Localization and Mobility Changes upon Induction of Nucleolar Reorganization by Drug Treatment**

Overexpression of a nonPP1 binding RRP1B variant induces morphological changes in nucleoli and leads to a reduction in 5-fluorouridine incorporation (Figure 3E). Moreover, RRP1B colocalizes with B23 and Pescadillo (Figure 4A). Taken together these observations suggest a role for PP1-RRP1B in rRNA metabolism. To investigate this further, we treated cells with actinomycin D (ActD) at low levels (0.5 µg/ml) to inhibit RNA Pol I and at high levels (2.5 µg/ml) to inhibit RNA Pol I and II. Both endogenous (Figure 5A, top panels) and GFP-tagged RRP1B (Figure 5A, bottom panels) relocates to the nucleoplasm in response to ActD and accumulate in small nucleolar foci of unknown function (Figure 5A, hashed arrows). This relocalization occurs more quickly in response to higher levels of ActD. A pool of nucleolar RRP1B is retained in the remnant central body (Figure 5A, arrows) and perinucleolar region. This peripheral nucleolar accumulation does not overlap markers for the well-characterized fibrillar in and coll caps (Supplemental Figure 2E) (Shay-Tal et al., 2005).

The nucleoplasmic redistribution of RRP1B was confirmed by cell fractionation and Western blot analysis (Figure 5B). In untreated cells, RRP1B is predominantly nucleolar (~80% of total protein found in this structure). The remaining signal is nucleoplasmic, and little or no RRP1B is found in the cytoplasm. On inhibition of transcription with ActD treatment, the nucleolar signal falls to ~50% total protein as a pool of RRP1B relocates to the nucleoplasm. There is little or no change in the cytoplasmic fraction.

Clearly, RRP1B localization responds to chemical treatment influencing RNA Pol I functionality. To discriminate between a potential role for RRP1B in rRNA transcription or rRNA processing, we exploited the unique properties of the nucleoside analog 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). DRB inhibits certain protein kinases, including casein kinase II (CK2), and indirectly inhibits RNA Pol II transcription. DRB reversibly dissociates rRNA transcription from later processing steps, causing segregation of nucleolar components into transcriptional “beads” and separate processing bodies (Scheer et al., 1984). Despite the breakdown in nucleolar structure, RNA Pol I transcription continues, with rRNA transcripts originating in the transcriptional beads and diffusing to the neighboring processing bodies. On treatment of U2OS cells with DRB, both endogenous and GFP-RRP1B relocate to smaller granular bodies (Figure 5A, arrows) surrounded by dispersed masses (Figure 5A, arrowheads). The relocation of core nucleolar RRP1B to the nucleoplasm upon DRB treatment was also demonstrated by Western blot analysis (Figure 5B). On removal of DRB, nucleoli reform and RRP1B and GFP-RRP1B resume their normal GC localization pattern (Supplemental Figure 3A). Coexpression of RRP1B and markers for either transcription/early processing (fibrillar in, Supplemental Figure 2F) or later rRNA processing (RPPL27, Supplemental Figure 2D) con-
firmed that the RRP1B granular bodies are indeed processing bodies, distinct from the transcriptional “beads.”

The association of RRP1B with rRNA transcripts is supported by the observation that the protein remains associated with the major nucleolar RNA signal after both ActD and DRB treatment. As in untreated cells, this localization is sensitive to RNAse treatment (Supplemental Figure 1, C and D). While ActD and DRB treatment led to relocalization of RRP1B-containing processing complexes within nuclei, we surmise that this is caused by different signal transduction events because the primary targets of both chemicals are distinct biomolecular complexes. Using a Fluorescence Recovery After Photobleaching (FRAP) approach, we measured the dynamic turnover of nucleolar GFP-RRP1B in untreated cells compared with cells treated with either ActD or DRB. The kinetics of recovery of a photobleached GFP-tagged protein can reflect the degree and affinity of its association with other proteins and/or nucleic acids. While both drug treatments induce reorganization of the nucleolus, ActD induces a significant ($p < 0.01$) decrease in the mobile fraction of nucleolar GFP-RRP1B ($17.3 \pm 0.82\%$ for Act D vs. $79.6 \pm 1.0\%$ for untreated; Figure 5C). Conversely, the nucleolar pool of GFP-RRP1B after DRB treatment has a similar mobile fraction to that observed in untreated cells, but the recovery rate is increased when compared with untreated cells (Figure 5C). These differences in the dynamic turnover rate of GFP-RRP1B between untreated, ActD-, and DRB-treated cells reveals distinct underlying interaction profiles. Their precise nature awaits investigation.

**RRP1B Coprecipitates 60S Ribosomal Subunit Processing Complexes**

To identify the molecular complexes with which RRP1B associates, we first carried out a quantitative SILAC-based immunoprecipitation (Figure 6) of GFP-RRP1B from U2OS-GFP-RRP1B-derived nuclear extracts. The most highly enriched interaction partner was Nol1, a known pre-60S (large) ribosomal subunit processing protein, followed by several other 60S processing proteins. We also noted a specific enrichment of the ribosomal proteins associated with the RPLs over the
small/40S ribosomal subunit proteins (RPSs) (full dataset presented in Supplemental Table 1). When coexpressed in cells, GFP-RRP1B and mCh-Nol1 colocalize throughout the entire cell cycle, supporting their presence in overlapping molecular complexes (Supplemental Figure 2A). A quantitative immunoprecipitation of the nonPP1 binding mutant GFP-RRP1B-KATA revealed a similar binding profile to that of GFP-RRP1B, with the obvious lack of PP1 (data not shown).

Although the GFP-RRP1B immunoprecipitation experiment identified RRP1B as a factor present in pre-60S ribosome subunit processing complexes, a caveat of overexpressed exogenous proteins is that they may not behave identically to the
endogenous protein. Thus, we used our RRP1B antibody for immunodepletion of the endogenous protein and carried out a quantitative proteomic screen of endogenous nuclear RRP1B. As shown in Figure 6C, endogenous RRP1B, like GFP-RRP1B, specifically enriches a large pool of RPLs (36/40 of the total) and an even more comprehensive array of known pre-60S processing proteins. Significant overlap was found between the interactomes of endogenous RRP1B and GFP-No1 (quantitatively immunoprecipitated from U2OS/CFP-No1-derived nuclear extracts; data not shown), confirming that these proteins are found in similar complexes. When we highlighted pre-60S ribosomal subunit-related proteins in our nucleolar GFP-PP1γ interactome, we confirmed both the enrichment of RRP1B and of these particular factors (Figure 6D), indicating that pulldown of nucleolar PP1 enriches a subset of pre-60S ribosomal subunit processing complexes to which it is targeted by RRP1B. Consistent with the plurifunctional nature of this enzyme, the nucleolar PP1 interactome also contains additional phosphatase complexes not involved in pre-60S ribosome biogenesis.

The quantitative aspect of these experiments, including built-in negative controls, combined with stringent applications and the application of “bead proteomes” to flag potential false positives (see Materials & Methods) (Trinkle-Mulcahy et al., 2008), provides a high level of confidence in the protein interaction partners identified. Nevertheless, we felt it important to confirm the interaction of RRP1B with a representative subset of proteins identified in our proteomic screen, including PP1β and PP1γ (Figure 1), No1 and B23 (Figure 7A), two RPLs (Figure 7B) and the pre-60S processing factor fibrillarin (Figure 7B). We also confirmed that RRP1B coprecipitates NNP-1/Nop52, indicating that both of these NOP52 domain-containing proteins are likely present in pre-60S processing complexes. This is intriguing as both are putative mammalian orthologues of yeast Rrp1p.

We further present a set of negative controls (i.e., proteins which, based upon their lack of enrichment in our RRP1B proteomic screens, should not be part of the RRP1B interactome). Indeed, Western blot analyses show that the small (405) ribosomal subunit protein RPS23 and the H/ACA snoRNP protein Gar1 do not coprecipitate RRP1B (Figure 7B). Furthermore, the RNA Pol I-related proteins RPA39 and RRN3, absent from our RRP1B interactome studies, do not coprecipitate RRP1B, again confirming the predicted role for RRP1B in later rRNA processing steps. Lastly, the 60S processing protein PWP1, which we found enriched with PP1 but not RRP1B, coprecipitated little to no endogenous RRP1B (Figure 7B). It should be noted that the small amount of RRP1B could be due to the difference in approach, as antibodies can be more sensitive than the mass spectrometer, and trace amounts of PWP1 may indeed be present in the RRP1B interactome.

Because our results suggest RRP1B may play a role in 60S processing, we wanted to examine the overlap between our RRP1B interactome and that of pre-60S processing complexes. The latter has not yet been studied to any great extent in mammalian cells, and thus we turned to the comprehensive 60S ribosomal subunit processing complex defined by detailed interactome studies in baker’s yeast system (Lebreton et al., 2008). We identified 66 mammalian orthologues to the 72 known yeast proteins depicted in this interaction diagram, of which 74% were found to be enriched with RRP1B (Figure 8A, yellow), representing a significant overlap between the yeast 60S processing complex and the mammalian RRP1B interactome. Having also noted a clear enrichment of RRP1B and other pre-60S processing proteins in the nucleolar PP1γ interactome, we compared this dataset to the yeast 60S processing complex and found enrichment of the full RRP1B interactome plus 8 additional proteins (Figure 8A, green). Proteins that were detected with PP1 but not RRP1B may be PP1-specific interaction partners but could also simply reflect differences in experimental conditions.

RRP1B is one of two suggested human orthologues for S. cerevisiae Rrp1p, a known rRNA processing protein. A direct comparison between the RRP1B and Rrp1p interactomes, the latter derived from a previous nonquantitative proteomic screen of Rrp1p (Horsey et al., 2004), revealed an overlap of 24 pre-60S processing proteins (not including RPLs) (Figure 8B). This confirms that RRP1B is found in similar complexes to its yeast counterpart. Note that the other human counterpart to Rrp1p, Nop52/NNP-1/hRRP1 (see below), is also part of the RRP1B interactome (Figure 8B).

As these interactomes represent a “snapshot” of pre-60S processing complexes at various stages of maturation, the fact that later processing and export factors are not found in either interactome suggests that the RRP1B/PP1 complex functions primarily at upstream stages of pre-60S subunit processing.

We next examined the spatial interaction of RRP1B with pre-60S ribosomal subunits, which are processed in the nucleolus and transit through the nucleoplasm to their final sites of action as mature ribosomes in the cytoplasm. In the U2OS/CFP-RPL27 cell line, the 60S ribosomal subunit marker GFP-RPL27 is distributed throughout the nucleolus, nucleoplasm and cytoplasm (Figure 7C, Supplemental Figure 2B), whereas RRP1B is predominantly enriched in nucleoli, with an additional ~20% found in the nucleoplasm (Figure 7C, Figure 3A). When GFP-RPL27 was depleted from these three subcellular fractions, it coprecipitated RRP1B from nucleolar and nucleoplasmic, but not cytoplasmic, fractions (Figure 7C). Similarly, PP1γ coprecipitated with GFP-RPL27 from these fractions. No detectable PP1γ was found with cytoplasmic 60S subunits, although a significant pool of the phosphatase is found in this subcellular compartment.

Finally, sucrose gradient fractionation confirmed the specific association of RRP1B with nuclear pre-60S (and not pre-40S) subunits (Figure 7D). PP1γ is found in both fractions, which fits with our identification of both RPs (Figure 6D) and 40S processing proteins such as MPP10 in the nucleolar PP1 interactome. Taken together, these data suggest that PP1 is targeted to and regulates multiple steps in the ribosome biogenesis pathway (see diagram in Figure 7E).

**RRP1B Is an Ancient Protein**

The NOP52 domain was defined as a conserved region within a nucleolar protein of 52 kDa. Cloning of this human autoantigen revealed it to be a protein previously named novel Nuclear Protein of 52 kDa (NNP-1 or hNop52). This protein has a high sequence identity/similarity to the S. cerevisiae rRNA processing protein Rrp1p, a protein involved in the maturation of the 275 rRNA (Savino et al., 1999) suggested that this NOP52 domain-containing protein (NNP-1) was the human homologue of S. cerevisiae Rrp1p and thus renamed this gene/protein as hRRP1.

KIAA0179 was the second human NOP52 domain containing protein to be identified and was thus designated RRP1B. Here we have identified RRP1B as a PP1 binding partner that docks via its RVxF motif. To further explore the architecture and possible origins of KIAA0179/RRP1B we performed a bioinformatic analysis of the sequenced genomes of a broad range of organisms. Using truncations of RRP1B and a Hidden Markov model (HMM) for the NOP52 domain (PFAM website), we identified and aligned all NOP52 domain-containing proteins and used three indepen-
dent methods (Neighbor Joining, Maximum Likelihood, Maximum Parsimony) to generate phylogenetic trees. Because primary sequences of RRP1B homologues are highly divergent, apart from the NOP52 domains, trees were built with the NOP52 domain sequences only. This yielded similar relationship patterns for all three methods.

The result of this search strategy and phylogenetic analysis is presented in Figure 9. Consistent with the NOP52 domain having been classified as a nucleolar domain of eukaryotic origin (Savino et al., 1999), we did not identify NOP52 proteins in Bacteria or Archeae. Although the NOP52 domain is highly conserved between KIAA0179/RRP1B and NNP-1/RRP1, phylogenetic analysis groups these proteins into separate branches when organisms contain both of these genes (NNP-1/RRP1 sequences are shaded blue). This analysis revealed the striking feature that the NNP-1/RRP1 protein appears only in mammals and, through gene duplication, has proliferated in several mammalian species. KIAA0179/RRP1B, on the other hand, is found in organisms throughout the eukaryotic lineage and is typically present as a single copy, with the exception of the genomes of P. tetraurelia (Chromalveolate), P. putens (spikemoss), and G.
max (bean). This suggests that KIAA0179/RRP1B is an ancient protein, from which the mammalian NNP-1/RRP1 likely derived, and that the rRNA processing function accredited to the NOP52 domain is an early eukaryotic event.

DISCUSSION

Ribosome subunit biogenesis is the major cellular function carried out by the nucleolus, with the very structure of this organelle intrinsically related to ongoing RNA Pol I transcription and pre-rRNA processing. Reports have also linked the nucleolus to control of a wide range of cellular pathways including cell division and DNA damage response (Pederson, 1998; Boisvert et al., 2007). It is now believed to be a major stress sensor, responding to stresses such as hypoxia and DNA damage by coordinating inhibition of ribosome biogenesis, cell cycle arrest, and, in certain cases, triggering of apoptosis (Mayer and Grummt, 2005). However, many of the regulatory events underlying these key functions remain undefined. Here we identified, via quantitative proteomics, fluorescence imaging and biochemical approaches, a nucleolar pool of PP1-RRP1B that is a component of pre-ribosomal subunit processing complexes. These results confirm our previous observations that a pool of PP1 activity accumulates within nucleoli and accounts for a large fraction of the associated Ser/Thr dephosphorylation events (Trinkle-Mulcahy et al., 2003).

Figure 8. RRP1B and PP1γ copurify an overlapping subset of pre-60S ribosomal subunit processing proteins. (A) An overlay of mammalian orthologues on their yeast counterparts in a yeast-derived map of pre-60S ribosomal subunit processing proteins demonstrates a specific enrichment of midlate processing complexes. Of the 72 proteins predicted in the yeast map, 66 have known mammalian orthologues. Of these, 49 (74%) were found in both the RRP1B and PP1γ interactomes (yellow), with an additional eight proteins only detected with the phosphatase (green). This may represent different subcomplexes or a difference in sensitivity of the experiment. Of the nine proteins that were not enriched (gray), most are peripheral to the core complex and function later in the processing/export pathway. In addition to these nonribosomal processing proteins, 36/40 (90%) of 60S subunit RPL proteins were specifically enriched with RRP1B and PP1γ. (B) 60S processing proteins identified in a recent nonquantitative screen of TAP-tagged yeast Rrp1p are indicated in this table, which includes both the yeast and mammalian gene names for each protein and uses the same color coding as in A to indicate whether a protein was found in our RRP1B and/or PP1γ IP or neither.
cleolar protein extraction method developed specifically for interactome analyses, we demonstrated the selective enrichment of a range of multiprotein complexes with nucleolar PP1 (Chamousset et al., 2010). Identifying RRP1B as an in-

Figure 9. Phylogenetic tree of NOP52 domain-containing sequences. A rectangular cladogram was generated by comparison of conserved regions in NOP52-domain containing sequences. Multiple phylogenetic tree inferences were performed (see Materials and Methods). Tree topology shown is NJ (1000 replicates) which is largely representative for all three methods. Most notable discrepancy was M. brevicolis, which aligns with the euglenozoa (MP) or animalia (ML) in other methods. Bootstrap values are indicated at the nodes. K, presence of a conserved (RRP1B/KIAA0179) PP1 interaction motif; F, presence of a FF doublet at C-terminal end.
interaction partner for nucleolar-targeted PP1β and PP1γ was not surprising as it has long been suggested as a candidate, although the interaction had not been confirmed (Moorhead et al., 2008; Hendrickx et al., 2009). Our biochemical studies and the dominant negative effect of RRP1B\textsubscript{KATA} overexpression conclusively validate RRP1B as a PP1 interactor.

Our initial hypothesis, namely that PP1 forms more than one complex in the nucleolus, is corroborated by several observations. The first is the enrichment of additional known/putative targeting subunits and a wide range of multiprotein complexes in the nucleolar PP1 interactome studies (Chamousset et al., 2010). Another is the presence of PP1 in both pre-40S and pre-60S peaks, with PP1-RRP1B limited to pre-60S peaks. Lastly, the limited dominant-negative effect of RRP1B\textsubscript{KATA} mutant overexpression suggests that other functional nucleolar PP1 complexes are not affected by its specific displacement from pre-60S ribosomal subunits. This work thus opens novel routes to elucidate the impact of PP1 on ribosome biogenesis via a direct approach on individual complexes.

The name RRP1B derives from the protein’s homology to the yeast Rrp1p protein (a.k.a. NOP52), which is involved in generation of 27S rRNA (Horsey et al., 2004). Nevertheless, the first published functional study on RRP1B identified it as a new candidate susceptibility gene for breast cancer progression and metastasis (Crawford et al., 2007). These data suggested that the protein plays a key role in regulating cell growth and proliferation, which could be a consequence of its predicted role in ribosome subunit biogenesis. More recently, RRP1B has also been linked to regulation of E2F-mediated apoptosis, and is believed to function directly in transcriptional control (Paik et al., 2010). The cellular role(s) of RRP1B thus remains open for debate, particularly in light of the existence of a second mammalian orthologue of NOP52, RRP1/NNP-1/Nop52 (Savino et al., 1999).

We provide here the first evidence for a role for RRP1B in 60S ribosome processing and also demonstrate that this protein has a functional PP1 interaction motif lacking in RRP1/NNP-1/Nop52. It is likely that RRP1B is the more complex cellular effector, as it can recruit a phosphoregulatory mechanism to a specific subset of processing complexes in the ribosome biogenesis pathway. It is also noteworthy that predicted RNA binding motifs display a different pattern between RRP1 and RRP1B, which may reflect differing RNA affinities. These differences will likely have significant impact on their respective cellular interaction profiles and functions. Our phylogenetic analysis indicates that RRP1/NNP-1/Nop52 and KIAA0179/RRP1B are both homologues of yeast Rrp1p, yet KIAA0179/RRP1B is the true functional orthologue. This is consistent with the significant overlap between our endogenous nuclear RRP1B interactome and recently identified TAP-tagged yeast Rrp1p interaction partners (Horsey et al., 2004). This does not exclude RRP1/NNP-1/Nop52 from a role in pre-60S subunit processing, and indeed it is also found in the RRP1B and PP1 interactomes, however it does raise the question of how much, if any, functional overlap exists between these two mammalian proteins with regard to pre-60S ribosomal subunit processing.

We have shown here that the subnucleolar targeting of RRP1B throughout the cell cycle coincides with that of several GC-localized pre-60S processing proteins, consistent with a role for RRP1B in nucleolar rRNA processing. This targeting requires rRNA transcripts but not the presence of rDNA. In contrast, fibrillarin, which is a DFC protein involved in earlier processing steps, is partially lost with RNase treatment and fully lost with DNase treatment, reflecting its association with both rDNA and rRNA (Ochs et al., 1985). This again suggests that RRP1B is mainly involved in rRNA processing, likely interacting with these molecules after they are released from sites of transcription.

We also exploited the well-characterized segregation of nucleolar components in response to actinomycin D and DRB treatment (Scheer et al., 1984; Louvet et al., 2006) to compare RRP1B behavior to that of both rRNA transcripts and other known rRNA processing proteins. The retention of a pool of RRP1B in the remnant central body of the nucleolus was unique compared with the loss of nucleolar B23 and the “capping” of fibrillarin at the nucleolar periphery. Interestingly, the pool of RRP1B that is lost to the nucleoplasm was found to accumulate in small foci. Further work will be necessary to define these foci and determine whether they represent a link to the suggested transcriptional role of RRP1B (Paik et al., 2010). With regard to the pool of nucleolar-retained RRP1B, it remains associated with both Pyronin Y-stained nucleolar RNA and GFP-RPL27 (Supplemental Figures 1C and 2C), again suggesting a structure/function relationship between RRP1B and nucleolar pre-60S processing complexes. Nucleolar-retained GFP-RRP1B in actinomycin D-treated cells is significantly less mobile than GFP-RRP1B in untreated cells, and as this pool of protein is also lost upon RNase treatment, it may be sequestered or “trapped” in inactive RNA processing complexes when ribosome biogenesis shuts down (Supplemental Figure 1C).

In contrast, DRB treatment, which induces segregation of FC, DFC, and GC constituents into a characteristic “beads on a string” conformation while preserving RNA Pol I activity, leads to a small but significant increase in the mobility of nucleolar GFP-RRP1B. The mechanism of action of DRB is still debated, as it is a CK2 inhibitor that also indirectly inhibits RNA Pol II. Furthermore, different DRB derivatives (DMAT, TBB) give contradictory results with regard to GFP-RRP1B dynamics (Supplemental Figure 3B). Finally, CK2 targets several key nucleolar proteins, including B23 and nucleolin, and the kinase has been postulated to play a crucial role in compartmentation of nucleolar protein complexes (Louvet et al., 2006). Thus, apart from experimental differences including cell type, concentration, and treatment time, and the off-target effects of kinase inhibitors that can complicate interpretation of results (Bain et al., 2007), ribosome biogenesis has such a plethora of potential CK2-dependent effectors that direct and indirect effects are difficult to tease apart.

Our localization data strongly supported a role for RRP1B in later stages of ribosome subunit biogenesis. To validate this hypothesis, we quantitatively defined the nuclear interactome of RRP1B. We indeed found an enrichment of large ribosomal proteins (RPLs) and proteins linked to processing and maturation and nuclear export are distinctly lacking in the RRP1B and PP1γ interactomes. Previously, we noted that the RRP1B\textsubscript{KATA} interactome is very similar to the wild-type RRP1B interactome. This suggests that RRP1B presence at rRNA processing complexes occurs independently of PP1, making RRP1B a bona fide PP1 targeting subunit.

Strikingly, proteins involved in very late stages of ribosome maturation and nuclear export are distinctly lacking in the RRP1B and PP1γ interactomes. Using stably expressed GFP-RPL27 as a marker for 60S subunits, we confirmed that association of RRP1B and PP1γ with these complexes occurs in both the nucleolus and nucleoplasm. Mature cytoplasmic 60S subunits, however, do not coprecipitate either RRP1B or
PP1, placing the site of action of this holoenzyme complex firmly within the nucleus.

Rrp1/Nop52 is an essential gene in yeast, with knockout compromising cell growth (Horsey et al., 2004). When we reduced cellular levels of RRP1B in U2OS or HeLa cells by ~90% using an siRNA approach (Supplemental Figure 4, B and D), little or no effect on cell growth or proliferation was evident. Although no significant changes were observed in the distribution of either nuclear pre-ribosomal subunits or mature cytoplasmic ribosomal subunits (data not shown), an increase in larger RNA species detected by Northern blot analysis may suggest an analogous role to its yeast counterpart in 28S processing (Supplemental Figure 4C). A similar lack of effect on cell growth or proliferation in response to reduction of RRP1B levels in human cells was observed recently by another group (Paik et al., 2010). This may reflect either a degree of genetic redundancy, or that levels of RRP1B are kept deliberately high to ensure it is never limiting for the essential process of ribosome subunit production and cell growth. Alternatively, subtle effects may be masked by the high level of ribosome biogenesis in immortalized cell lines and/or the presence of RRP1/NNP-1/Nop52. Alternate approaches, such as analysis in primary cell lines or concurrent knockdown of RRP1B and RRP1/NNP-1, will be required to better understand the functional role of RRP1B-PP1 in regulation of pre-60S processing and, potentially, coordination of this pathway with regulation of transcription and/or proliferation.

Importantly, future work must focus on identification of RRP1B-PP1 targets within the pre-60S processing complex. Known phosphoproteins such as B2S and Elf6 are likely targets, with the latter being particularly attractive given that dephosphorylated pre-60S subunit-bound Elf6 is believed to prevent premature association of 40S and 60S subunits in the nucleus (Ceci et al., 2003). It is also important to further characterize proteins identified in the RRP1B nuclear interactome that are not directly related to pre-60S processing, as they may represent links to pathways that are controlled concurrently with ribosome biogenesis.

As discussed here, the ubiquitous nature of PP1 in cellular regulation emphasizes the importance of identifying and characterizing the specific holoenzyme complex(es) involved in each pathway. As part of our systematic dissection of the molecular mechanisms controlling targeting of PP1 activity to nucleolar substrates in ribosome biogenesis, cellular proliferation, and stress response pathways, we have identified and characterized a major nucleolar pool of PP1 targeted to pre-60S ribosomal subunit processing complexes by RRP1B. It is anticipated that this information will lead to a more direct therapeutic intervention by facilitating the targeted disruption of PP1 activity in disease states related to nucleolar dysfunction, including cancer, accelerated aging and viral infection.

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