The U4/U6 Recycling Factor SART3 Has Histone Chaperone Activity and Associates with USP15 to Regulate H2B Deubiquitination*

Received for publication, January 20, 2014, and in revised form, February 12, 2014. Published, JBC Papers in Press, February 13, 2014, DOI 10.1074/jbc.M114.551754

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Background: Ubiquitin conjugation and deconjugation on histone H2B regulate transcription and pre-mRNA splicing.

Results: The splicing factor SART3 binds histones and enhances deubiquitination of H2B by the deubiquitinating enzyme, Usp15.

Conclusion: SART3 is a dedicated histone chaperone that cooperates with Usp15 to deubiquitinate free histones.

Significance: The coordinated activities of Usp15 and SART3 provide a link between H2B deubiquitination and pre-mRNA splicing.

Post-translational modifications of histone proteins produce dynamic signals that regulate the structure and function of chromatin. Mono-ubiquitination of H2B in the histone tail (at Lys-123 in yeast or Lys-120 in humans) is a conserved modification that has been implicated in the regulation of transcription, replication, and DNA repair processes. In a search for direct effectors of ubH2B, we identified a deubiquitinating enzyme, Usp15, through affinity purification with a nonhydrolyzable ubH2B mimic. In the nucleus, Usp15 indirectly associates with the ubH2B E3 ligase, RNF20/RNF40, and directly associates with a component of the splicing machinery, SART3 (also known as TIP110 or p110). These physical interactions place Usp15 in the vicinity of actively transcribed DNA. Importantly, we found that SART3 has previously unrecognized histone chaperone activities. SART3, but not the well-characterized histone chaperone Nap1, enhances Usp15 binding to ubH2B and facilitates deubiquitination of ubH2B in free histones but not in nucleosomes. These results suggest that SART3 recruits ubH2B, which may be evicted from DNA during transcription, for deubiquitination by Usp15. In light of the function played by SART3 in U4/U6 di-snRNP formation, our discovery points to a direct link between eviction-coupled erasure of the ubiquitin mark from ubH2B and co-transcriptional pre-mRNA splicing.

In eukaryotes, the state of chromatin affects all processes that require access to DNA, such as transcription, replication, and DNA repair. DNA accessibility is regulated in part by numerous post-translational modifications (PTMs) on histones; these include acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, and sumoylation. The reversibility of these covalent modifications and their combinatorial occurrence contribute to their function as highly diverse and dynamic signals (1, 2). In comparison to most other PTMs, ubiquitination stands out because of its considerable size. Ubiquitin (Ub) is a protein of 76 amino acids with a well-folded and highly stable structure. It is attached to other proteins through the formation of an isopeptide linkage between the C-terminal carboxylate of Ub and a lysine side chain of the substrate protein. In addition, Ub can be attached to other Ub molecules to form poly-Ub chains (3). Although all four core histones and linker histone H1 have been reported to undergo ubiquitination at various positions, the predominant ubiquitinated histones in humans are H2A ubiquitinated at Lys-119 and H2B ubiquitinated at Lys-120 (hereafter referred to as ubH2A and ubH2B) (4). Mono-ubiquitination of histones H2A or H2B does not lead to their degradation, but instead serves as a non-proteolytic signal that regulates gene expression and DNA repair processes. Interestingly, the same modification on different histones is associated with opposite effects: whereas ubH2A is associated with gene silencing, ubH2B is implicated primarily in active transcription (5).

ubH2B is conserved from yeast to man (4). In humans, it is catalyzed by the RNF20/RNF40 heterodimeric E3 ligase, which interacts with the PAF complex and rides with the RNA polymerase II (RNAPII) in transcription elongation (6–8). Consistently, genome-wide localization studies suggest that ubH2B is largely associated with actively transcribed genes (9–11). However, how ubH2B regulates transcription elongation has been enigmatic. Multiple observations that link ubH2B to various
steps of the elongation process include: 1) ubH2B stimulates H3K4 di- and tri-methylation as well as H3K79 di- and trimethylation (12, 13); 2) ubH2B inhibits compaction of nucleosomal arrays in vitro (14); 3) ubH2B co-operates with the histone chaperone FACT and remodeling enzyme Chd1 to reassemble nucleosomes in the wake of elongating RNAPII (15–17); 4) ubH2B inhibits recruitment of elongation factors, such as Ctk1 in yeast (18) and TFIIS in humans (19), to impede elongation. Inhibitory functions of ubH2B in promoter regions have also been reported (20, 21). That ubH2B can affect transcription both positively and negatively underscores the importance of ubH2B dynamics. Several deubiquitinating enzymes (DUBs) have been reported that target ubH2B (5). The best-characterized histone DUB, the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex, is conserved throughout eukaryotes and has important roles in transcription and mRNA export (22, 23). Recent reports that ubH2B is enriched at the intron-exon boundaries in yeast, flies and humans suggest a novel link between ubH2B and co-transcriptional pre-mRNA splicing (10, 11). In yeast, deletion of the ubH2B ligase has mild splicing defects but exhibits synthetic sickness with components of the U1, U2, and U5 snRNPs (11, 24). Whether and how ubH2B regulates splicing directly or indirectly is unclear.

We have developed a method to synthesize nonhydrolyzable mimics of ubH2A and ubH2B in large quantities. Using these proteins in pull-down assays from nuclear extracts, we identified a DUB, Usp15, which binds to ubiquitinated histones with high affinity. We also found that Usp15 associates indirectly with the ubH2B ligase RNF20/RNF40 and directly with a component of the splicing machinery, SART3. Our characterization of the Usp15/SART3/ubH2B interactions points to a direct link between H2B deubiquitination and co-transcriptional pre-mRNA splicing.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—The coding sequences of Usp15, Usp15NTD-(1–222), Usp15CTD-(256–952), Usp4, and SART3, were cloned into the pcDNA5 FRT/TO vector (Invitrogen) downstream of a tandem Flag-Flag-HA epitope tag. Site-directed mutagenesis was used to generate all point mutations. The following plasmids were used for bacterial expression: pMal-C2 Usp15, pGEX4T-2 Usp15-His6, pGEX4T-2 Usp4-His6, pET21a Flag-HA-H2A, pET21a Flag-HA-H2B, pET21a H2A(K119C), pET21a H2B(K120C), and pET3a His6-Ub(G76C). For expression in Sf21 insect cells, the coding sequences of Usp15, Usp11 were purchased from Dharmacon.

**Cell Culture, Transfections, and Treatments**—DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamax, 100 units/ml Pen/Strep (Invitrogen) was used to culture HeLa, HEK293, and their derivatives. DMEM/F-12 (1:1) supplemented with 2.5 mM 1-glutamine, 10% FBS, 15 mM Heps-KOH, pH 7.5, and antibiotics as described above was used to culture hTERT-RPE-1 cells. Stable cell lines were generated with pcDNA5 FRT/TO constructs using the parental Flp-In T-REx cell lines (Invitrogen) as described by Yao et al. (25). Doxycycline at 1 μg/ml was routinely used to induce target gene expression. SF21 cells were cultured at 27 °C in SF900-II SFM medium according to ATCC guidelines.

DNA and siRNA were transfected with Lipofectamine 2000 or Lipofectamine RNAiMax (Invitrogen), respectively, according to manufacturer’s instructions. Depletion of RNAi target was assayed 72 h post-transfection by reverse transcription (iScript, Bio-Rad) and real-time PCR (SYBR Green Supermix, Bio-Rad), or by immunoblotting. siRNA sequences used are: Usp4 (5′-CGAAGAAUGGAGAGGAACAUU-3′), RNF20 (5′-GAAGGACGUUGUAGAGAU-3′), SART3 #1 (5′-GGAGCAGGAAUCCCUA-3′), and SART3 #2 (5′-GAUGUGCU-GUCGCAAGUU-3′). siGenome pools against Usp15 and Usp11 were purchased from Dharmacon.

For cycloheximide chase experiments, cells were treated with 100 μg/ml cycloheximide 72 h after siRNA transfection. At indicated times, cells were harvested in SDS-lysing buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS), and target protein levels were analyzed by SDS-PAGE and quantitative immunoblotting. The same procedure was employed for experiments with the E1 inhibitor, except that cells were treated with 10 μM E1 inhibitor. Quantitative immunoblots used fluorescent secondary antibodies and were analyzed with a Li-COR Odyssey scanner and Image Studio Software.

**Recombinant Proteins**—For recombinant proteins expressed in bacteria, BL21(DE3) *Escherichia coli* bearing the expression plasmid were grown to log phase and expression was induced with 0.4 mM IPTG at 37 °C for 3 h. The only exception was MBP-Usp15, whose expression was induced at 19 °C for 16 h before harvesting. **GST-Usp15-His6 purification:** cell pellets were resuspended in PBS containing 1% Tween-20 and PIC (protease inhibitor mixture: 1 mM PMSF, 50 μM TLCK, 1 μg/ml pepstatin, 1 μg/ml leupeptin), and lysed by sonication. Affinity purification with glutathione-agarose (GE Healthcare) was performed according to manufacturer’s instructions. The eluate was further purified with Ni-NTA agarose (Qiagen) following manufacturer’s instructions. Final elutions containing >90% full-length Usp15 were dialyzed into PBS, 5 mM βME (β-mercaptoethanol), 10% glycerol, and stored at −80 °C in aliquots. MBP and MBP-Usp15 purification: cell pellets were lysed in 50 mM Tris, pH 7.6, 300 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 0.01 mg/ml DNase I, 5 mM βME; affinity purification with amylose resin (New England Biolabs) were done according to manufa-

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4 GenBank Accession Number NM_006313.2.
5 GenBank Accession Number NM_199443.2.
6 GenBank Accession Number NM_014706.3.
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cturer’s instructions. Eluates containing purified proteins were dialyzed against 20 mM Tris, pH 7.6, 50 mM NaCl, 5 mM βME, 10% glycerol. MBP-Usp15 was further purified on a Mono Q column using a 0.05–0.8 M linear gradient of NaCl. His6-Ub(G76C) was purified using Ni-NTA agarose according to manufacturer’s protocol except that 5 mM βME was added in all buffers. Eluates were dialyzed against 10 mM Tris, pH 8.0, 50 mM NaCl, 0.2 mM EDTA, 10 mM βME, and then passed through Q Sepharose Fast Flow resin (GE Healthcare). His6-Ub(G76C) remained in the flow-through, which was dialyzed into 1 mM HOAc prior to lyophilization. All recombinant histones were expressed and purified according to Dyer et al. (26). Those that contained the cysteine substitution were dialyzed into 1 mM HOAc in the final step prior to lyophilization. His6-Usp2cc and His6-Nap1 were purified as described (27, 28).

His6-Flag-SART3 was expressed in Sf21 insect cells using the BacPAK baculovirus expression system (Clontech). 72 h post-infection with recombinant virus, cells were harvested, and recombinant SART3 was enriched with Ni-NTA-agarose. Eluates from the Ni-NTA column were dialyzed against 20 mM Tris, pH 7.6, 50 mM NaCl, 5 mM βME, followed by further purification on a Mono Q 5/50 GL column (GE Healthcare) by HPLC. A linear gradient of 0.05–0.8 M NaCl in 20 mM Tris, pH 7.6, 5 mM βME, 10% glycerol was applied and SART3 was eluted at 450 mM NaCl. To eliminate contaminating DUB activities that co-purified with SART3, peak fractions from the Mono Q column were diluted to lower the salt to 200 mM, incubated with 1.7 μM Ub-vinyl sulfone (Ub-VS; BostonBiochem) for 2 h at 37 °C, and then passed through a Superdex 200 column to remove excess Ub-VS. The Ub-VS-treated SART3 did not affect Ub-AMC hydrolysis by Usp15 (data not shown).

Histone Dimer, Octamer, and Mono-nucleosome Reconstitution—147 bp DNA containing 601 positioning sequence were prepared according to Dyer et al. (26). Purified recombinant human H2A, H2B (or semi-synthetic ubH2B), H3.3, and H4 were assembled into octamers and further purified on a Superdex 200 column. H2A/H2B dimers were assembled similarly and further purified on a Superdex 75 column. Canonical mono-nucleosomes were reconstituted by salt dialysis (26) and ubH2B-containing nucleosomes were prepared by salt dilution according to McGinty et al. (29). Acid-extracted histones were prepared as described by Rogakou et al. except that acid-soluble proteins were dialyzed against water to allow refolding of histones (30). To prepare native nucleosomal substrates, HEK293 cells were lysed in hypotonic buffer (20 mM Tris, pH 8.0, 10 mM KCl, 1 mM MgCl₂, 0.3% Nonidet P-40, 30 mM N-ethylmethylamino-lic, and PIC) on ice for 10 min and centrifuged to collect nuclei. Nuclei were resuspended at 1 × 10⁸ cells/ml in hypotonic buffer supplemented with 420 mM NaCl. Nuclei were again pelleted and washed twice in MNase (micrococcal nuclease) digestion buffer (50 mM Tris, pH 8, 10 mM CaCl₂, 2 mM MgCl₂). Digestion was performed with 80 units/ml MNase at 25 °C for 30 min, and then the soluble fraction was collected and digested again with 20 units/ml MNase to achieve homogeneous mono-nucleosome products.

Semi-synthesis of Native ubH2B—The coding sequence of H2B-(1–116) was cloned into pTXB1 vector (New England Biolabs) between Ndel and Sap1 sites. The resulting plasmid was transformed into ER2566 cells (New England Biolabs) and expression of H2B-(1–116)-intein fusion was induced with 0.5 mM IPTG at 25 °C for 6 h. Cells were harvested and lysed in lysis buffer (50 mM Tris, pH 7.6, 200 mM NaCl, 1 mM EDTA, supplemented with 10 mM MgCl₂, 20 μg/ml DNase I, and PIC). Cleared lysates were incubated with chitin beads at 4 °C overnight, followed by extensive wash of the beads with lysis buffer. To generate H2B-(1–116)-α-thioester, beads were incubated with lysis buffer containing 50 mM mercaptoethanesulfonate at 4 °C overnight, followed by elution with SAU buffer (20 mM NaOAc, pH 5.2, 7 μM urea). Eluates were further purified on a Mono S column by HPLC. Fractions containing >90% H2B-(1–116) were pooled and presence of the thioester was confirmed by MALDI-TOF mass spectrometry. Subsequent synthesis follows the scheme as reported previously (31).

Supercoiling Assay—Supercoiling assays were performed as described previously (32) except that pGEM3Z plasmid DNA was used instead of pBR322, and the final products were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining.

Immunofluorescence—Cells were cultured on coverslips and fixed with 2.5% paraformaldehyde at 37 °C for 10 min. After washing with PBS, cells were permeabilized in wash buffer (PBS, 0.1% Triton X-100) at 25 °C for 20 min. This was followed by blocking in PBS, 5% BSA, 0.1% Triton X-100, 2 mM MgCl₂ for 10 min, and incubations with primary and secondary antibodies with washes inbetween. Cells were then stained with DAPI (1 μg/ml) for 5 min before coverslips were mounted to glass slides with ProLong Gold antifade reagent (Invitrogen). For staining using ubH2B antibody (Cell Signaling), cells were fixed and permeabilized as described above. Now, chromatin was unwound by treating with 2 M HCl in PBS for 10 min at 37 °C. The remainder of the staining protocol was as described above except PBS was used for all wash steps.

Generation of Cross-linked Ub*Histone Mimics—Cross-linking reactions adopted the procedure described by Yin et al. (33). Lyophilized His6-Ub(G76C), H2A(K119C), or H2B(K120C) was resuspended in 10 mM HOAc, 7 μM urea at 10 mg/ml. Histone and Ub were at a 2:1 molar ratio in 6 M urea, 50 mM sodium borate, pH 8.5, 5 mM TCEP (Tris(carboxyethyl)phosphine). The cross-linker, 1,3-dichloroaceton, was added at a molar concentration equal to half of the sum of all free cysteines in the reaction. After incubation on ice for 30 min, the reaction was stopped with 5 mM βME. To remove unreacted histones, the mixture was diluted 1:10 in denaturing binding buffer (50 mM NaPi, pH 8, 300 mM NaCl, 6 mM urea, 10 mM imidazole, 5 mM βME) and incubated with Ni-NTA agarose. After washing with the same buffer, the bound fraction, which contained Ub, Ub*Ub, and Ub*histones, was eluted with the binding buffer plus 250 mM imidazole. Eluate was used to reconstitute either histone dimers or octamers directly. Ub and Ub*Ub were removed during the subsequent gel filtration step.

To generate ub*GSH (Ub cross-linked to glutathione), a 1:5 molar ratio of His6-Ub(G76C) to glutathione was used to perform the cross-linking as described above, except that urea was omitted from the reaction. The reaction mixture was loaded on a Mono S 5/50 column in 50 mM ammonium acetate, pH 5.5, 5 mM βME, and proteins were eluted with a linear gradient of 0–1
M NaCl in the same buffer. Peak fractions containing ub*GSH were pooled and purified further with the same column. The ub*GSH product was confirmed by MALDI-TOF mass spectrometry (data not shown).

Deubiquitination Assays—For deubiquitination of Ub-AMC (BostonBiochem), reactions were performed in assay buffer (50 mM Hapes, pH 7.5, 0.5 mM EDTA, 0.5 mg/ml BSA, 200 mM NaCl, 5 mM DTT) with 2 nM enzyme unless otherwise indicated; 0.05% Brij-35 was added to reactions containing SART3. In competition assays, competitor proteins were added to an enzyme-containing master mix, and reactions were initiated by addition of the substrate. Ub-AMC hydrolysis was monitored continuously for 1 h at 30 °C on a fluorescence plate reader (BioTek Synergy 4, λ ex = 340 nm and λ em = 440 nm); initial velocities of fluorescence increases were converted to [AMC] release per min by reference to an AMC standard. Data were fitted with the Michaelis-Menten equation and, for competition assays, with a one-site binding model using PRISM (GraphPad Software).

Qualitative deubiquitinating assays using native or semi-synthetic ubH2B substrates were done at 37 °C for indicated times in reaction buffer (10 mM Hapes, pH 7.5, 200 mM NaCl, 0.05% Brij-35, 25 μM ZnSO 4 , 5 mM DTT, 0.5 mg/ml BSA).

RESULTS

Usp15 Binds to Mono-ubiquitinated Histone H2A or H2B in Vitro—We aimed to identify proteins that bind preferentially to either unmodified, ubH2A-containing, or ubH2B-containing histone dimers. To avoid heterogeneity from PTMs that naturally occur on histones prepared from cells, we adopted a strategy previously used to synthesize nonhydrolyzable di-Ub analogues (33) and ubiquitinated PCNA (34) from recombinant proteins. Because there are no native cysteines in Ub, H2A, or H2B, we used site-directed mutagenesis to incorporate a cysteine at the C terminus of Ub (UbG76C) and the primary ubiquitination site on H2A or H2B (H2A-K119C and H2B-K120C, respectively). Each mutant protein was expressed in E. coli and purified. Ub was then cross-linked to each histone with a bifunctional thiol cross-linker, dichloroacetone (Fig. 1A). The resulting cross-link is one C-C bond longer than an isopeptide linkage and bears an additional carboxylate group, but has the advantage that it is highly stable and not susceptible to deubiquitination. Purified ub*H2A or ub*H2B (* denotes the cross-link) was assembled with Flag-tagged H2B or H2A into histone dimers (Fig. 1B) and used in pull-down assays to isolate interacting proteins from HeLa cell nuclear extract. Proteins bound to unmodified or ubiquitinated dimers under stringent conditions (470 mM NaCl) were identified by mass spectrometry. Among the few proteins that preferentially bound to ub*H2A or ub*H2B-containing histone dimers, we identified Usp15. Immunoblotting analysis confirmed that Usp15 bound only to ubiquitinated histone dimers (i.e. ub*H2A/H2B or H2A/ub*H2B), but not to Ub alone or unmodified H2A/H2B (Fig. 1C). Nap1, a well characterized histone chaperone, was used as a control as it showed no preference for unmodified or ubiquitinated histone dimers (35).

To quantify the affinity and specificity of the interactions between Usp15 and the ubiquitinated histones, we used the nonhydrolyzable ub*histone mimics in DUB activity assays as potential inhibitors of deubiquitination. Purified recombinant Usp15 can hydrolyze a general DUB substrate composed of Ub conjugated to 7-amino-4-methylcoumarin (Ub-AMC). We determined that Usp15 has a K m of 1.15 ± 0.017 μM and a k cat of 5.12 ± 0.29 min -1 with this substrate (data not shown). Upon addition of ub*H2A/H2B or H2A/ub*H2B dimers, Ub-AMC hydrolysis by Usp15 was inhibited in a dose-dependent manner (Fig. 1D). In contrast, similar concentrations of unmodified H2A/H2B dimers did not inhibit Usp15 significantly. To address whether the artificial cross-link mediates unexpected interactions with the DUB, we used dichloroacetone to cross-link UbG76C to glutathione. Whereas the IC 50 values for inhibition by the ub*histone mimics were each ~0.1 μM, the IC 50 for ub*GSH was nearly 60-fold higher (Fig. 1D). Further analysis showed that inhibition of Usp15 by H2A/ub*H2B was best described using a competitive binding model with a K i of 34 nM (Fig. 1E). These results demonstrate that the interactions between Usp15 and ubiquitinated histones are direct, specific, and of high affinity. Because H2A/ub*H2B did not affect Ub-AMC hydrolysis by the Usp2 catalytic core domain (Usp2cc) (Fig. 1D) (36), this behavior is not a property shared among the USP family of DUBs.

Usp15 Deubiquitimates Free and Nucleosomal Histones with a Strong Preference for Free Histones—To test directly whether Usp15 deubiquititates free histones or nucleosomes, we prepared acid-extracted histones from HEK293 cells and mononucleosomes purified from micrococcal nuclease-digested chromatin. As a positive control, we used the recombinant DUB module of the yeast SAGA complex (SAGA DUB ), which is known to deubiquitinate H2B in vivo and in vitro (37, 38). Fig. 2A shows that both Usp15 and SAGA DUB deubiquitinated H2B as free histones or in nucleosomes. Because histones and nucleosomes purified from cells are heterogeneous, we sought to obtain a chemically-defined substrate. Using a strategy that combined solid phase peptide synthesis and native chemical ligation (31) we obtained ubH2B that has a native Ub-histone isopeptide linkage and is free of other post-translational modifications (Fig. 2B) (31, 39). This ubH2B was assembled with recombinant human H2A, H3.3, and H4 histones into octamers or mono-nucleosomes (Fig. 2C). Using these homogeneous substrates, we found that both Usp15 and SAGA DUB deubiquitinated H2B in the context of histone octamers or mono-nucleosomes (Fig. 2D). However, with either the cell-derived or semi-synthetic substrates, Usp15 preferentially deubiquitinated the histones whereas SAGA DUB preferred the nucleosomes. Recombinant Usp15 and SAGA DUB had similar activities against nucleosomal substrates, but Usp15 was ~10-fold more active against the histone substrates (Fig. 2E).

Usp15 Indirectly Associates with the ubH2B Ligase RNF20/RNF40—Previously, RNF40 was identified by mass spectrometry as a potential Usp15-associated protein (40). To avoid the overexpression conditions used in that study, we established an inducible stable cell line that expresses Flag-tagged Usp15 at endogenous levels in the absence of doxycycline (Dox) due to leaky expression (Fig. 3A). We found that, with or without overexpression, both endogenous RNF20 and RNF40 co-immunoprecipitated with Usp15 (Fig. 3B). To examine if Usp15 has a
role in the regulation of RNF20/RNF40 stability, we depleted Usp15 and its paralog, Usp4, in HeLa cells by siRNA-mediated knockdown and used cycloheximide-chase to monitor the half-lives of the E3s (Fig. 3, C and D). In cells treated with control siRNA, both RNF20 and RNF40 were highly stable and showed no detectable changes of protein levels during the chase. Depletion of Usp15 and Usp4 did not affect RNF20 stability but did accelerate turnover of RNF40. The effect on RNF40 half-life was moderate, but reproducible. Therefore, Usp15 and Usp4 play only a minor role in protecting the E3 ligase RNF40 from degradation, possibly by counteracting its auto-ubiquitination. When we co-expressed RNF20, RNF40 and Usp15 in insect cells, despite robust expression, we failed to detect significant association between Usp15 and the RNF20/RNF40 dimer (data...
Therefore, Usp15’s association with RNF20/RNF40 most likely is indirect.

**Usp15 Directly Associates with the U4/U6 Recycling Factor SART3**—Human Usp15 has 73% sequence similarity with another DUB, Usp4. Both DUBs are comprised of two well-defined protein domains: the NTD contains a DUSP (domain specific for USP) and a UBL (Ub-like) domain, whereas the CTD contains the catalytic residues (Fig. 4A). Previously, Song et al. reported that Usp4 plays a role in facilitating pre-mRNA splicing through its interactions with SART3 (41). In the early stage of spliceosome assembly, SART3 promotes the formation of the U4/U6 di-snRNP (42, 43). As the spliceosome matures, the U4 snRNP is ejected, bound by SART3, and then recycled for the next round of assembly. Usp4 was shown to deubiquitinate Prp3, a component of the U4 snRNP, in order to facilitate the ejection of U4 and formation of the U2/U5/U6 active splic-
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Because of the high homology between Usp15 and Usp4, we examined the association between Usp15 and SART3. Usp15 co-immunoprecipitates via its NTD with endogenous SART3 (Fig. 4B). Reciprocally, SART3 co-immunoprecipitates with endogenous Usp15 (Fig. 4C). As a splicing factor, SART3 is exclusively nuclear with a small fraction in puncta that have been reported to be Cajal bodies (44). When we examined the cellular localization of Usp15, we found that endogenous Usp15 predominantly is in the cytoplasm with a small but clearly detectable fraction that is nuclear. Interestingly, Usp15 NTD or CTD expressed independently are primarily nuclear (Fig. 4D), suggesting that intramolecular interactions within Usp15 and its interaction with SART3 modulate its localization.

The Usp15-SART3 interaction is direct, as purified recombinant Usp15 and SART3 bind to each other in vitro (Fig. 4F). Using a ligand-depletion assay, we estimated that the binding between the Usp15 NTD and SART3 has a $K_d$ of 42 nM (Fig. 4G).

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FIGURE 3. Usp15 associates with RNF20/RNF40 and regulates RNF40 stability. A, an inducible stable cell line that expresses Flag-Usp15 in response to doxycycline (Dox). The Flag-Usp15 inducible stable cells were treated with or without 1 $\mu$g/ml Dox for 48 h, and cell lysates were analyzed by SDS-PAGE and immunoblotting along with parental cell lysates for comparison. In the absence of Dox, Flag-Usp15 was expressed at a similar level as endogenous Usp15 (compare lane 1 and 2 on the anti-Usp15 blot). Note that anti-Usp15 detects both endogenous and Flag-tagged Usp15, which migrate the same on this gel. B, endogenous RNF20 and RNF40 co-immunoprecipitate with Usp15. Extracts were prepared from the parental or Flag-Usp15-expressing cells with or without Dox treatment, incubated with anti-Flag agarose, and the bound proteins were detected by SDS-PAGE and immunoblotting with the indicated antibodies. Asterisk denotes a nonspecific band. C, Usp4 and Usp15 depletion did not affect protein stability of RNF20 and moderately reduced the stability of RNF40. HeLa cells were transfected with 6.6 nm Control siRNA or a combination of Usp4 and Usp15 siRNAs at 3.3 nm each. 72 h post-transfection, cells were treated with cycloheximide (CHX) for the indicated times prior to harvesting. Protein levels were monitored by SDS-PAGE and quantitative immunoblotting. A representative experiment is shown. D, quantitation of blots shown in C. Tubulin levels were used for normalization. Shown are the average changes (fold) from three independent experiments. Error bars represent standard deviations.
that under the high salt condition of the nuclear extract, SART3 no longer associated with Usp15; thus, the association between SART3 and H2A/H2B is independent of Usp15 and insensitive to Ub modification of the histones. Because SART3 and some of its associated proteins interact with RNA, we treated the nuclear extract with RNase A and repeated the pull-down experiments; the RNase A treatment had no effect on SART3-H2A/H2B binding (Fig. 5B).

Using purified recombinant proteins, we found that SART3 not only directly binds to H2A/H2B dimers, but also to H3/H4 tetramers and histone octamers (Fig. 5C). In contrast, we observed no binding to mono-nucleosomes. These properties suggest that SART3 has histone chaperone-like activities. Classic histone chaperones, such as Nap1, promote nucleosome assembly by disaggregating free histones to allow productive histone-DNA interactions (45). This nucleosome assembly process requires the deubiquitination of histones.
activity can be tested in an assay in which nucleosome formation on relaxed circular DNA leads to supercoiling (46). Using this assay, we found that SART3 promoted nucleosome formation in a dose-dependent manner (Fig. 5D). In comparison with Nap1, SART3’s ability to promote supercoiling is much weaker, which suggests that the physiological function of SART3 may not be to promote nucleosome assembly on a large scale. However, we cannot rule out that certain histone PTMs or other associating factors may enhance nucleosome assembly by SART3, or that SART3 might facilitate assembly at specific sites on chromatin.

**In vitro** SART3 did not affect Ub-AMC hydrolysis by Usp15 (Fig. 6A). However, the H2A/ub*H2B dimer was a much stronger inhibitor of Usp15 in the presence of SART3, suggesting that SART3 could play a role in substrate recruitment (Fig. 6B). Moreover, in contrast to SART3, addition of the Nap1 histone chaperone abolished the inhibition by H2A/ub*H2B. In experiments where we assayed for a direct, positive effect of SART3 on Usp15 activity, we found consistently that SART3 enhanced deubiquitination of ubH2B in the context of histone octamers, whereas Nap1 inhibited (Fig. 6C). When ubH2B-containing nucleosomes were used as substrates, SART3 had no effect on deubiquitination by Usp15 (Fig. 6D). These data strongly suggest that SART3 is a specialized histone chaperone that helps to recruit free-histone substrates to Usp15.

**SART3 Regulates Global ubH2B Levels**—Although Usp15 binds to ubiquitinated histones with high affinity and efficiently deubiquitinates them in vitro, knockdown of Usp15 alone or in combination with Usp4 did not affect global ubH2A or ubH2B levels (Fig. 7A). One possibility is that other cellular DUBs have redundant functions in histone deubiquitination. However, to our surprise, depletion of SART3 resulted in a dramatic increase in ubH2B levels but that ubH2A levels were unchanged (Fig. 7B). The increase in ubH2B was not caused by increased

*FIGURE 5. SART3 has histone chaperone activity.* A, SART3 binds to unmodified and ubiquitinated H2A/H2B dimers. Flag-tagged Ub, H2A/H2B, or ubiquitinated H2A/H2B were used in pull-down assays as described in Fig. 1C except that NaCl concentration was adjusted to 300 mM in all steps. Note that Flag-H2A ran as a doublet on the gel when the bound fractions had been concentrated by TCA precipitation prior to SDS-PAGE. B, RNA does not contribute to SART3-histone interactions. Flag-tagged Ub or H2A/H2B dimers were used in pull-down assays as described in A. Where indicated, nuclear extracts were treated with 20 µg/ml RNase A prior to incubation with the immobilized proteins. C, SART3 binds to H2A/H2B, H3/H4, and histone octamers, but not to mono-nucleosomes. Purified recombinant Flag-SART3 was immobilized on anti-Flag agarose beads and incubated with 2 µM H2A/H2B, 0.5 µM H3/H4, 0.5 µM histone octamers, or 0.5 µM mono-nucleosomes at 37 °C for 1 h. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie staining. Anti-Flag agarose (“beads”) was used as a negative control. D, SART3 facilitates nucleosome assembly. Supercoiling assays were performed with 0.3 µM Nap1 or increasing amounts of SART3 (0.14, 0.29, and 0.57 µM). Nucleosome assembly on the plasmid DNA was measured by formation of supercoils that promote faster migration on the gel.
levels of either RNF20 or RNF40. Also, knockdown of SART3 did not affect Usp15 protein levels. When we examined other histone modifications associated with active transcription, we found no changes in H3K36me3 or H3K4me3 levels. However, the increase of ubH2B was accompanied by a small increase in H3K79me2. Although both H3K4me3 and H3K79me2 depend on ubH2B (12, 47), H3K4me3 primarily localizes to promoters whereas H3K79me2 is in ORFs. Genome-wide, ubH2B localization correlates poorly with H3K4me3, but strongly with H3K79me2 (10). These observations suggest that the increase in ubH2B upon SART3 knockdown most likely occurs in gene bodies, which is consistent with the role of SART3 in co-transcriptional splicing.

When we examined ubH2B levels by immunofluorescence, we found that ubH2B was increased in almost all of the cells that had SART3 depleted (Fig. 7C and D), ruling out that this is a cell cycle-related phenotype. Because genetic and biochemical evidence have established a strong link between H2B ubiquitination and transcription elongation (48), we also examined the global pool of actively transcribing RNAPII. In SART3-depleted cells, we found no changes in RNAPII CTD Ser-2 or Ser-5 phosphorylation, which are hallmarks of the elongating polymerase (Fig. 7E). The effect of SART3 knockdown on ubH2B levels was observed in HeLa cells (Fig. 7F), MD-MBA-231 breast cancer cells (Fig. 7A) and non-cancerous hTERT-RPE-1 cells (Fig. 7F). Furthermore, depletion of SART3 with two different siRNA oligonucleotides each led to increased ubH2B (Fig. 7F). Conversely, Dox-induced overexpression of SART3 in a stable cell line decreased ubH2B levels (Fig. 7G). These data suggest a direct role of SART3 in the regulation of ubH2B.

To deconvolute the possible contributions of SART3 to ubH2B conjugation and deconjugation, we treated cells with a highly specific E1 inhibitor (49) to block Ub conjugation and monitored ubH2A and ubH2B levels over time. Both ubH2A and ubH2B levels decreased over the course of 1 h after E1 inhibition (Fig. 7H and I). Whereas ubH2B half-life was 15–20 min in both Control siRNA-treated and non-transfected cells, SART3 knockdown resulted in a doubling of the ubH2B half-life to 35 min. In contrast, no effect on the ubH2A half-life was observed. Knockdown of Usp15 and Usp4 did not affect the half-lives of either ubH2A or ubH2B (data not shown). These data show that SART3 plays an important role in H2B deubiquitination. Although we cannot exclude other DUBs from being involved, our pull-down results and in vitro characterization strongly implicate Usp15 in this process.

**DISCUSSION**

Eukaryotic transcription requires the coordination of complex machineries that 1) execute initiation, elongation and termination; 2) create access to DNA and restore chromatin after the passage of RNAPII; and 3) process and deliver mRNA to the translation machinery. Current evidence suggests that a highly conserved histone PTM, H2B ubiquitination, lies at the crossroads of transcription elongation, mRNA processing, and chromatin restoration. How this modification supports these functions is uncertain and complex. Recent work that utilized ubH2B-containing nucleosomal arrays to identify proteins that preferentially interact with uniformly ubiquitinated chromatin identified some of the players in ubH2B function (50). However, whether H2A/H2B dimers or nucleosome complexes are the substrates for Ub attachment or removal in vivo is an open
FIGURE 7. SART3 regulates global ubH2B levels. A, depletion of SART3, but not Usp4 and Usp15, increases ubH2B levels. MDA-MB-231 cells were transfected with 6.6 nM Control or SART3 siRNA, or a combination of Usp4 and Usp15 siRNAs at 3.3 nM each. 72 h post-transfection cell lysates were harvested and analyzed by SDS-PAGE and immunoblotting. Tubulin was used as a loading control. Slices of immunoblots that came from the same gel are boxed together. ubH2A and ubH2B were detected on the same blot with monoclonal rabbit anti-ubH2A (Cell Signaling) and mouse anti-ubH2B (MediaMab) antibodies and two-color fluorescent secondary antibodies.

B, HeLa cells were transfected with 3.3 nM Control or SART3 siRNA; 72 h post-transfection, protein levels were monitored as described in A.

C, Global ubH2B increase in SART3-depleted cells. HeLa cells transfected with Control, RNF20, or SART3 siRNA were stained with anti-ubH2B antibodies and DAPI. Depletion of RNF20 abolished almost all ubH2B staining, whereas depletion of SART3 led to increased ubH2B signals ubiquitously.

D, fluorescence intensities of ubH2B signals from 100 cells in each condition as described in C were quantitated using SlideBook Reader. Error bars represent standard deviations. Using a paired Student’s t test, p values of < 0.0001 were calculated for SART3 or RNF20 knockdown in comparison with the Control knockdown.

E, depletion of SART3 did not change RNAPII CTD phosphorylation levels globally. HeLa cells were transfected with Control or SART3 siRNA; 72 h post-transfection, protein levels were monitored as described in A.

F, hTERT-RPE-1 cells were transfected with 3.3 nM Control siRNA or two different siRNAs that target SART3; 72 h post-transfection protein levels were monitored by SDS-PAGE and immunoblotting.

G, overexpression of SART3 decreases global ubH2B levels. Cell lysates were prepared from parental or Flag-SART3 stable cells with or without Dox treatment, and protein levels were monitored as described in A. H–I, SART3 depletion affects ubH2B deconjugation. HeLa cells were transfected with Control or SART3 siRNA as described in A. 72 h post-transfection, 10 μM E1 inhibitor was added and cell lysates harvested at the indicated times. Protein levels were monitored by quantitative immunoblotting with fluorescent secondary antibodies and normalized to H2B levels. Changes in ubH2B and ubH2A levels were plotted relative to the value at time 0 for each condition.
question. Ubiquitination of H2B is known to accompany transcription, a process during which nucleosomes are disassembled or remodeled to allow access for transcription activators and RNAPII; following passage of the transcribing RNAPII, evicted histones are reassembled into nucleosomes to prevent unwanted access to DNA. H2A/H2B dimers clearly are obligatory intermediates in this process, but their states with regard to ubiquitination or other PTMs are not well characterized.

The Specificity of Histone Deubiquitination—In mammals, the PRC1 and RNF20/RNF40 complexes catalyze Ub conjugation to H2A Lys-119 and H2B Lys-120, respectively (5). In contrast, many histone DUBs identified to date are promiscuous toward these functionally distinct substrates. SAGA\textsubscript{DUB}, Usp44, and Usp3 have all been reported to deubiquitinate both H2A and H2B (51–54), suggesting that targeting of these DUBs is the main factor in determining specificity. Usp15 binds to ub\textsuperscript{H2A} and ub\textsuperscript{H2B} mimics with similar affinities and deubiquititates both ub\textsuperscript{H2A} and ub\textsuperscript{H2B} efficiently (Figs. 1\textsuperscript{D} and 2\textsuperscript{A}), suggesting that it has no intrinsic specificity that differentiates these substrates. However, Usp15 association with RNF20/RNF40 and SART3 most likely places it at sites of active transcription and provides access to ub\textsuperscript{H2B} substrates.

Because ub\textsuperscript{H2A} and ub\textsuperscript{H2B} may exist both within and outside of nucleosomes, another aspect of DUB specificity is whether they differentiate nucleosomal from free Ub-histone conjugates. Based on qualitative assays, Usp16 and Usp49 appear to prefer nucleosomal ub\textsuperscript{H2A} and ub\textsuperscript{H2B}, respectively (55, 56). Usp15 is the first example of a DUB that prefers free histone substrates. With synthetic ub\textsuperscript{H2B} as a substrate, Usp15 showed a strong preference for ub\textsuperscript{H2B}-containing octamers, whereas SAGA\textsubscript{DUB} preferred nucleosomal ub\textsuperscript{H2B} (Fig. 2, D and E). Note that these DUB assays contained 200 mM NaCl; in that condition, the histone octamers most likely dissociate into H2A/H2B dimers and H3/H4 tetramers. SAGA\textsubscript{DUB} has a conserved arginine-rich surface whose mutation affects SAGA function in yeast (38). It was postulated that this surface may mediate interactions with nucleosomal DNA (37). Our data provide the first evidence for preferential deubiquitination of nucleosomal versus free histones by SAGA\textsubscript{DUB} and supports this idea. Usp15 by itself showed little specificity for binding to unmodified H2A/H2B dimers, yet it binds to ub\textsuperscript{H2A} or ub\textsuperscript{H2B}-containing dimers ~60-fold better than to Ub alone. This suggests that surfaces on histones and Ub might both contact Usp15, and that their combined effects contribute to specificity for Ub-histone conjugates.

In the cell, histones that are not in nucleosomes are most likely complexed with histone chaperones; thus, physiologically, Usp15 might never encounter free histone substrates. This is in fact suggested by our observation that Nap1, a generic histone chaperone, blocked Usp15 binding to ub\textsuperscript{H2B} (Fig. 6B). Our discovery that SART3 is a binding partner of Usp15 and has histone chaperone-like properties resolves this apparent conundrum. In contrast to Nap1, SART3 enhanced ub\textsuperscript{H2B} binding to Usp15 and also ub\textsuperscript{H2B} hydrolysis by Usp15 (Fig. 6, B and C). These data strongly argue that free histones, rather than nucleosomes, are the likely substrates for the Usp15/SART3 complex.

Usp15 has been implicated in the deubiquitination of a wide variety of substrates, including proteins in the TGF\beta-signaling pathway and several E3 ligases (57–62). Although Usp4 and Usp15 sequences are very similar, they most likely perform overlapping, but non-identical, functions. In nuclear extract, Usp15 but not Usp4 was identified by mass spectrometry as bound to ubiquitinated histones. This suggests that, of these two DUBs, Usp15 is selective for histone substrates. Distinct functions also are evident from experiments that showed depletion of Usp4, but not Usp15, leads to cell cycle defects (41), thus, Usp4 is primarily responsible for deubiquitination of the splicing factor Prp3. Knockdown of either or both DUBs failed to change appreciably global ub\textsuperscript{H2A} or ub\textsuperscript{H2B} levels. This could be due to existence of other DUBs that deubiquitinate H2A or H2B. Substantial redundancy has been observed for deubiquitination events associated with DNA double-strand break repair (52). Another possibility is that only a subset of ub\textsuperscript{H2A} or ub\textsuperscript{H2B} is regulated by Usp15 in specific cellular processes. Defining this subset is a goal of our future work.

SART3 Is a Histone Chaperone That Functions in Co-transcriptional Splicing—In eukaryotes, transcription and mRNA processing are tightly coupled; both are regulated by dynamic chromatin disassembly and reassembly. SART3 is a recycling factor that assists pre-mRNA splicing by promoting formation of the U4/U6 di-snRNP. Its homolog in yeast, PRP24, is an essential gene with similar recycling functions (63). Our discovery that SART3 binds to histones suggests that components of the splicing machinery may regulate aspects of chromatin dynamics. During transcription, capture by SART3 of displaced histones to limit exchange with the free histone pool could be one of these functions. An emerging concept is that not only does chromatin structure regulate the efficiency of splicing, but that the splicing machinery can provide feedback to regulate transcription machinery and chromatin (64, 65). Our biochemical results suggest that one function of SART3-histone interactions is to assist Usp15-mediated H2B deubiquitination. Evidence from multiple organisms indicates a conserved role for ub\textsuperscript{H2B} in regulating co-transcriptional pre-mRNA splicing. ub\textsuperscript{H2B} is enriched at the 5′-ends of actively transcribed genes and exon/intron boundaries (10, 11). In yeast, deletion of either BRE1 (H2B E3 ligase) or UBP8 (an H2B DUB) enhances the splicing defect of ribosomal protein genes when splicing is compromised by deletion of NPL3, which functions to promote recruitment of U1 and U2 snRNPs (66). Similarly, depletion of the BRE1 counterpart, RNF20, suppresses exon skipping in human cells (10). It remains unclear whether ub\textsuperscript{H2B} plays a direct role, such as recruiting splicing factors, or an indirect role in splicing through its function in transcription elongation. Our results now show that, in either case, the SART3-Usp15 complex is most likely responsible for erasing the Ub mark from histones that are evicted during transcription. This function may be particularly important to maintain low ub\textsuperscript{H2B} levels in introns. Because ub\textsuperscript{H2B} assists in nucleosome reassembly after RNAPII passage (16), erasure of the mark may help to maintain low nucleosome occupancy in introns, thereby enhancing the chromatin signatures that direct the next round of transcription and splicing (Fig. 8). A recent report identified another DUB, Usp49, that specifically deubiquitinates H2B in nucleo-
SART3 Binds USP15 and Regulates H2B Deubiquitination

A model for co-transcriptional ubH2B deconjugation by the SART3-Usp15 complex. The RNF20/RNF40 heterodimer associates with the PAF complex (PAF1C), which directly binds to RnapIIP. This complex catalyzes H2B ubiquitination and stimulates transcription elongation. SART3 and other components of the splicing machinery are recruited to process nascent transcripts co-transcriptionally. During this process, histones are displaced from nucleosomes to allow RnapIIP to access the DNA template. Ejected histones are bound by the SART3-Usp15 complex and Usp15 deconjugates Ub from histone dimers. Non-ubiquitinated histone dimers are now available for re-assembly into nucleosomes in the wake of elongating RnapIIP.

some (55). Loss of Usp49 leads to a decrease in splicing efficiency in a subset of genes. Among a limited set of genes that we tested, genes whose splicing are affected by Usp49 were not affected by SART3 knockdown. Therefore, it is likely that SART3-Usp15 and Usp49 have different sets of target genes.

Using an inhibitor of the E1 Ub-activating enzyme, we report for the first time that in HeLa cells ubH2A and ubH2B have half-lives of 40 min and 15 min, respectively (Fig. 7, H and I). The half-life of ubH2B is similar to what was observed upon inhibition of transcription (48), supporting the idea that ubH2B conjugation is largely co-transcriptional. At steady state, inhibition of transcription (48), supporting the idea that ubH2B conjugation is largely co-transcriptional. At steady state, 10–15% of H2A is ubiquitinated, whereas only 1–5% of H2B is ubiquitinated (4). Our data suggest that the faster turnover of ubH2B is at least in part responsible for its lower abundance. SART3 knockdown increased ubH2B half-life ~2-fold with no effect on ubH2A, yet knockdown of Usp15 or Usp4 had no effect on global ubH2B or ubH2A levels. At this point, it is unclear whether the ubH2B increase upon SART3 knockdown is a direct effect mediated by SART3-associated DUBs or if it is an indirect effect mediated by other genes whose expression depends on SART3. Very little is known about the genes that SART3 regulates directly; identification of these genes in the future will be required to rule out indirect effects.

Acknowledgments—We thank Michael Rape, Cynthia Wolberger, and the Protein Expression and Purification Facility at CSU for providing reagents for this work. The E1 inhibitor was kindly provided by Millennium: The Takeda Oncology Company. We also thank Robert E. Cohen for critically reading the manuscript and providing purified Usp2cc.

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