FEBS Letters 580 (2006) 6233–6241

The Polycomb-associated protein Rybp is a ubiquitin binding protein

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Received 26 July 2006; revised 27 September 2006; accepted 11 October 2006

Available online 19 October 2006

Edited by Felix Wieland

Abstract **The Rybp protein has been promoted as a Polycomb group (PcG)-associated protein, but its molecular function has remained elusive. Here we show that Rybp is a novel ubiquitin binding protein and is itself ubiquitinated. The Rybp interacting PcG protein Ring1B, a known ubiquitin E3 ligase, promotes Rybp ubiquitination. Moreover, one target of Rybp's ubiquitin binding domain appears to be ubiquitinated histone H2A; this histone is a substrate for Ring1B's E3 ligase activity in association with gene silencing processes. These findings on Rybp provide a further link between the ubiquitination system and PcG transcriptional repressors.**

Keywords: Rybp; Ring1B; Polycomb group; Ubiquitin; NZF; Histone H2A

1. Introduction

Post-translational modification of proteins through the attachment of chemical groups or small polypeptides has been implicated in the regulation of diverse cellular processes including signal transduction, intracellular transport, transcription, and DNA repair. The attachment of ubiquitin, a 76 amino acid polypeptide, to lysine residues of target proteins originally was described as a signal for proteasomal-mediated degradation (reviewed in [1,2]). However, it is now recognized that different modes of ubiquitin conjugation exist, including the attachment of lysine 48-, lysine 29-, lysine 63-, or lysine 6-linked polyubiquitin chains or the attachment of single moieties of ubiquitin (reviewed in [3–7]). While the lysine 48-linked and lysine 29-linked polyubiquitin chain attachments are most often correlated with protein degradation, other forms of ubiquitin attachment result in changes in function, interactions, and/or subcellular localization of the modified protein ([8,9]; see also [10,11]).

Ubiquitin conjugation is brought about through an enzymatic cascade involving an E1 activating enzyme, an E2 conjugating enzyme, and, in most cases, an E3 ligase that confers substrate specificity (reviewed recently in [1,2]). Ubiquitinated proteins are known to interact with adapter/receptor proteins that harbor domains capable of binding to the modifications in a non-covalent manner (reviewed recently in [12–17]). Several distinct types of ubiquitin binding domains (UBD) have been described including the UIM, CUE, UBA, UEV, PAZ, NZF, and others. Current models suggest that the adapter/receptor proteins containing these UBDs translate the ubiquitin signals of their target proteins by altering function, localization or stability (reviewed in [12–16,18]).

Here we assessed whether Rybp (for Ring1 and YY1 binding protein; also known as DEDAF), by virtue of its harboring a putative UBD in its amino terminal region, is able to function as a ubiquitin binding protein. Rybp first was isolated as an interacting partner for Polycomb group (PcG) proteins, specifically associating with those PcG proteins that have been assigned to the PcG PRC1 complex, including Ring1A, Ring1B (a.k.a. Rnf2), and M33 ([19]; for reviews on PcG complexes see [20–24]). Historically classified as transcriptional corepressors that function to establish a heritable repressed state of key developmental genes, PcG proteins are now implicated in the processes of proliferation, survival, self-renewal, and tumorigenesis (for reviews see [20–27]). On the molecular level, transcriptional regulation by PcG proteins is achieved in part through their abilities to post-translationally modify histones. For example, the PcG protein Ring1B has been reported to promote monoubiquitination of histone H2A, with this modification playing an important role in both X inactivation and Hox gene silencing [28–31]. The Ring1B-related Ring1A protein has been shown to stimulate the E3 ligase activity of Ring1B towards histone H2A, as has another Polycomb repressive group complex 1 (PRC1) member, Bmi1 ([31] also see [32,33]). The role of Rybp in PcG complexes has remained elusive, although it has gained support in vivo in a recent report on *Drosophila* Rybp. Specifically, transcriptional repression activity of dRybp is strictly dependent on the presence of PRC1-complex members [34]. Moreover, flies that lacked dRybp showed some homeotic transformations, a phenotype

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Abbreviations: CFP, cyan fluorescent protein; NZF, Npl4 zinc finger; ORF, open reading frame; PcG, Polycomb group; PRC1, Polycomb repressive group complex 1; TS, trophoblast stem; UBD, ubiquitin binding domain; uH2A, ubiquitinated histone H2A; YFP, yellow fluorescent protein

0014-5793/$32.00
doi:10.1016/j.febslet.2006.10.027

typically associated with disruption of key PcG components in vivo [34].

We now show that Rybp has an NZF UBD that mediates its interaction with ubiquitin and ubiquitinated proteins. This NZF also promotes the ubiquitination of Rybp itself, a modification that is enhanced by Ring1B. The integrity of Rybp's NZF appears to be important for the subnuclear localization of Rybp and Ring1B to Polycomb group bodies. Binding assays indicate that one target of Rybp's ubiquitin binding domain is ubiquitinated histone H2A. Consistent with this, Rybp, Ring1B, and ubiquitinated histone H2A can be found together on the inactive X chromosome in trophoblast stem cells. Together, these findings are in line with the emerging theory that Rybp may participate in PcG protein-mediated transcriptional repression (see also [19,34]), and suggest that this function for Rybp is tied to its role as a ubiquitin binding protein.

2. Materials and methods

2.1. Plasmid construction

Rybp cDNA was amplified by RT-PCR from total mouse prostate RNA and cloned in frame with a 3′ FLAG tag in pcDNA 3.1$^+$ or with a 5′ GST moiety in pGEX-5X-1. To generate mutations within the Rybp's NZF UBD (TF-AA mutation), the QuikChange Site Directed Mutagenesis Kit (Stratagene) was used with the following primers (with underlined mutations): GATTGTAGCGTCTGCGCCGCTAGGAACAGCGCCGAA (sense) and TTCGGCGCTGTTCCTAGCGGCGCAGACGCTACAATC (antisense). The Ring1A cDNA was amplified by RT-PCR from mouse liver RNA and was cloned in frame with a 5′ FLAG tag in pcDNA3.1$^+$. pcDNA-Myc-Ring1B was a gift from H. Koseki, RIKEN Yokohama Institute. PCR-generated Rybp or Rybp(TF-AA) were subcloned in frame into a pEYFP-C1 plasmid carrying a 5′ yellow fluorescent protein (YFP) sequence. PCR-generated Ring1B was subcloned in frame into a pECFP-C1 plasmid carrying a 5′ cyan fluorescent protein (CFP) sequence. pKH3-Myc-Ub was a gift from D. Germain, Mount Sinai School of Medicine). PCR-generated ubiquitin open reading frame (ORF) was cloned into pcDNA-His/C, in frame with a 5′ 6 × His tag. Further details about plasmid construction are available upon request.

2.2. Biosensor binding experiments

Biosensor binding experiments were performed at 20 °C using a BIACORE 2000 (Biacore AB, Uppsala, Sweden) equipped with a research-grade CM4 sensor chip. GST-fusions of the RYBP protein, the TF-AA mutant protein, and the Npl4-NZF domain were expressed in BL21(DE3) CodonPlus (Stratagene) cells using the auto-induction method [35]. Half liter cultures were started from single colonies and allowed to grow 10 h at 37 °C, and then switched to room temperature for growth to saturation (overnight). Cell pellets were lysed in 40 ml of lysis buffer (50 mM Tris 8.0, 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 5% glycerol and a cocktail of protease inhibitors (leupeptin, PMSF, aprotinin, and pepstatin), and then cleared of cell debris by centrifugation. About 3000 RU anti-GST Ab was immobilized to the CM4 chips using amine-coupling chemistry. GST-fusion proteins (or recombinant GST alone, reference) were captured directly on the antibody surfaces at densities of about 1000 RU from soluble *Escherichia coli* lysates diluted in running buffer (25 mM Tris pH 8.0, 150 mM NaCl, 5 mM βME, 10 μM $ZnCl_2$, 0.2 mg/ml BSA and 0.01% P20). Pure recombinant wildtype ubiquitin and the I44A mutant were expressed and purified as described previously [36], and injected in running buffer in triplicate over the three surfaces at concentrations of 0–1000 μM (50 μl/min). Data were collected at a rate of 2 Hz during the 25 s association and dissociation phases.

2.3. In vitro binding assays

For the ubiquitin binding assays, 25 μg GST-Rybp or GST-Rybp(TF-AA) were immobilized onto glutathione sepharose beads and incubated with 250 μg total protein extract from HeLa cells, either non-transfected or over-expressing Myc-tagged ubiquitin, in GST binding buffer (100 mM Tris pH 7.4, 200 mM NaCl, 1% NP-40, 5 mM βME, 1× protease inhibitor cocktail, Roche) or with binding buffer alone. Samples were incubated for 1 h at 4 °C, washed with binding buffer, and loaded onto SDS–PAGE followed by Western blotting analysis using an antibody directed against ubiquitin (1:100, U5279 SIGMA).

For the ubiquitinated histone H2A binding studies, nucleosomal histones were prepared by subcellular fractionation into nuclear extracts and nuclear pellets, and pellets were then resuspended in buffer C (20 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA) and briefly sonicated to bring nucleosomes in solution. Approximately 25 μg GST-Rybp or GST-Rybp(TF-AA) proteins were then incubated with 500 μg of histones in GST-binding buffer for one hour at 4 °C. Samples were then washed and subjected to Western blotting analysis using antibodies directed against histone H2A (1:500; 07-146, Upstate) or against ubiquityl-histone H2A, clone E6C5 (1:100; 05-678, Upstate).

2.4. Cell culture-based assays

HeLa cells were transfected with the indicated constructs using FuGene (Roche) or the calcium phosphate precipitation method. Forty-eight hours post-transfection cells were collected, washed with PBS, lysed in PG buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA and protease inhibitors), incubated on ice for 20 min and centrifuged.

For more stringent conditions in the in vivo ubiquitination assay, cells were lysed with 100 μl SDS buffer (1:3 ratio of Buffer 1: 5% SDS, 150 mM Tris pH 6.9, 30% glycerol, to Buffer 2: 25 mM Tris pH 8.3, 50 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), with protease inhibitors, and incubated on ice for 15 min. Lysates were sonicated twice on ice and then boiled for 2 min. Proteins were then diluted 1:10 with dilution buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF and protease inhibitors).

For immunoprecipitations, equal amounts of proteins were precleared for 20 min with 30 μl protein G agarose (Sigma), and FLAG-tagged proteins were immunoprecipitated for 90 min at 4 °C using 30 μl of agarose-conjugated anti-FLAG antibody (Sigma). Samples were washed with PG buffer (or dilution buffer for the high stringency assay) and then loaded onto SDS–PAGE for Western blot analysis using antibodies directed against Rybp/DEDAF (1:1000; AB3637 Chemicon), the Myc tag (1:100; OP10 Calbiochem), or the HA tag (1:300; sc-805 Santa Cruz).

2.5. Subcellular localization analysis

U2OS cells were grown on glass coverslips in 6-well dishes until 70% confluent and then transfected using FuGene with 100 ng of pEYFP/C1-Rybp or pEYFP/C1-Rybp(TF-AA) with or without pECFP/C1-Ring1B, following the manufacturer's instructions. Eighteen hours post-transfection, cells were washed twice with PBS, coverslips were removed from the wells and cells were fixed in 2% formaldehyde, 4% sucrose in PBS for 5 min. Coverslips were then washed twice with PBS and mounted on glass slides, and images were acquired using a CDD camera mounted on an Olympus 1×81 microscope. Images were then processed using IPLab software (Scanalytics). To determine the levels of expression of the fluorescent fusion proteins, the remaining cells in each transfected well were collected, lysed in PG buffer, and subjected to Western blotting analysis using antibodies directed against Rybp (1:1000; AB3637 Chemicon) or Ring1B (kind gift of Dr. H. Koseki, RIKEN Yokohama Institute).

For the trophoblast stem (TS) cell studies, XX TS cells (kind gift of K. Huynh and J. Lee, Harvard) were grown on irradiated mouse embryonic fibroblasts (MEFs) in TS medium (RPMI 1640 with 20% fetal bovine serum, 1 mM sodium pyruvate, 10 μM β-ME, 2 mM L-glutamine) plus 25 ng/ml FGF4 and 1 μg/ml heparin freshly added as previously described [37]. Preparation of TS cells for immunofluorescence analysis was performed as previously described [29]. Dilutions of primary antibodies were as follows: anti-Ring1B/RNF2 [38]; kind gift of H. Koseki, RIKEN Yokohama Institute) 1:2, anti-ubiquityl-Histone H2A, clone E6C5 (05–678, Upstate) 1:50, anti-RYBP (AB3637, Chemicon) 1:100. Secondary antibodies GAR IgG-568 1-11036 and GAM IgG-488 A-11029 from Molecular Probes were diluted 1:200, and DAM IgM-FITC 715-095-140 from Jackson ImmunoResearch Laboratories, Inc. was diluted 1:50. Immunofluorescence was recorded using a Zeiss AxioCam MRc Camera running MRGrab 1.0.0.4 on a Leica DMRB compound fluorescence microscope.

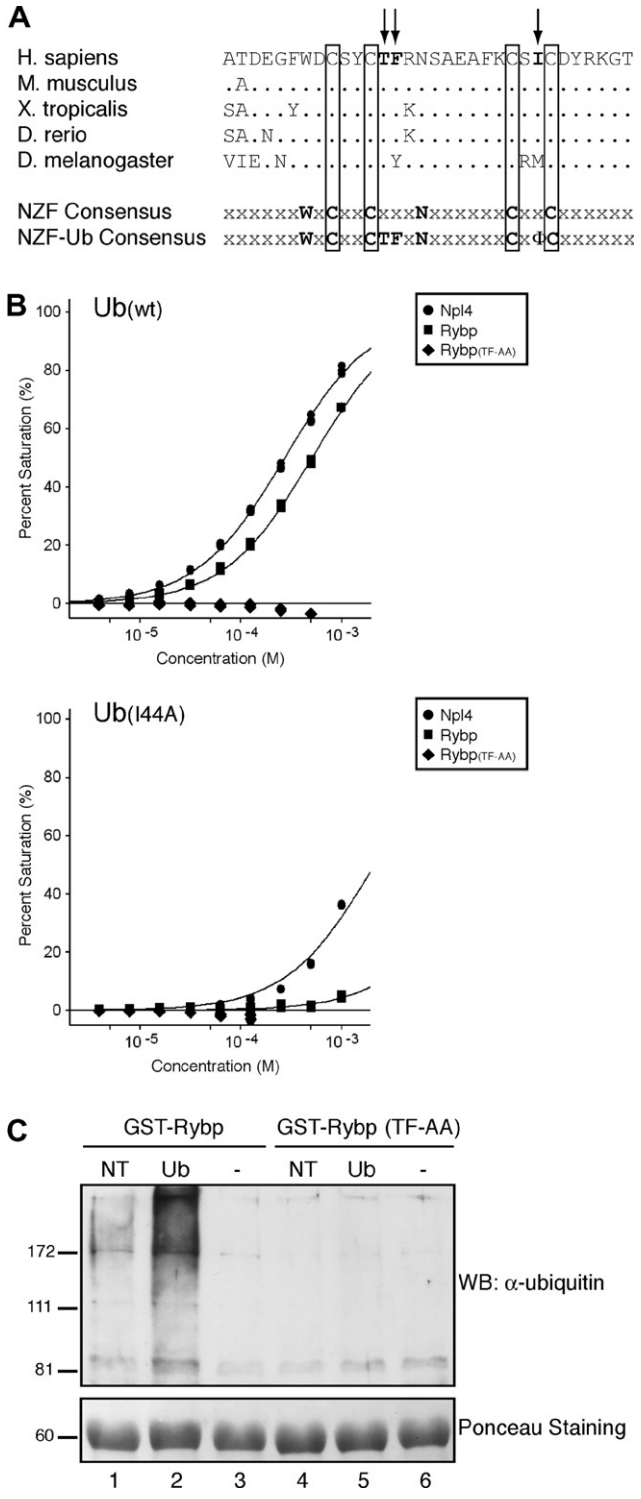

Fig. 1. Rybp can bind to ubiquitin and ubiquitinated proteins in vitro. (A) Aligned amino acid sequences of the NZF domains of several Rybp orthologs (*Homo sapiens*, NCBI Protein Database NP_036366: *Mus musculus*, NCBI Protein Database NP_062717; *Xenopus tropicalis*, NCBI Protein Database P_001016846; *Danio rerio* NCBI Protein Database NP_958474; *Drosophila melanogaster*, from FlyBase, gene CG12190). Consensus sequences for NZF motifs, as well as for the subset of NZF motifs that specifically recognize ubiquitin (Ub), are shown below. The zinc-coordinating cysteines are boxed, the amino acids that function as a molecular signature for NZF modules are in bold, and the critical residues of NZFs involved in ubiquitin binding are marked by arrows. Φ = hydrophobic residue. (B) Biosensor isotherms showing wildtype ubiquitin (top) or I44A-ubiquitin mutant (bottom) binding to immobilized GST-Rybp (squares), GST-Rybp(TF-AA) (diamonds) and GST-Npl4 NZF (circles, positive control). Dissociation constants and their errors were derived by fitting simple 1:1 models to the equilibrium binding data (solid lines) [39]. Background binding to a control GST surface was negligible (not shown). (C) Western blotting (WB) analysis of GST-pulldowns performed using GST-Rybp and GST-Rybp(TF-AA) and total protein extracts from HeLa cells either non-transfected (NT) or transfected with Myc-tagged ubiquitin (Ub). Recovered proteins were probed with an antibody directed against ubiquitin. Equal levels of GST-Rybp (lanes 1–3) and GST-Rybp(TF-AA) (lanes 4–6) were used in all samples, as indicated by Ponceau Staining (bottom panel). Molecular weight is shown in kDa on the left.

3. Results and discussion

3.1. Rybp harbors an NZF UBD with a dual function

Examination of Rybp's coding region sequence reveals the presence of a zinc finger of the NZF (for Npl4 zinc finger) type ($x(n)$–W–x–C–$x(2)$–C–$x(3)$–N–$x(6)$–C–$x(2)$–C–$x(n)$) (Fig. 1A). Recently it has been shown that a subset of NZF domains, specifically those containing a $_{13}TF_{14}/\Phi_{25}$ motif surrounding the zinc coordination site, possess ubiquitin binding activity [39–42]. Because the NZF $_{13}TF_{14}/\Phi_{25}$ motif is conserved in Rybp orthologs throughout evolution (Fig. 1A; see arrows indicating the $_{13}TF_{14}/\Phi_{25}$ residues; Φ = hydrophobic), we asked whether Rybp was indeed capable of ubiquitin binding in vitro. Biosensor experiments revealed that an immobilized GST-Rybp fusion protein was able to bind to free ubiquitin in solution with a dissociation constant of 479 ± 4 μM (Fig. 1B, upper graph, squares). Although relatively weak, this low affinity interaction is in the range of that reported for other ubiquitin binding domains (UBDs) (see for example the GST-Npl4 NZF control in Fig. 1B, upper graph, circles) and may facilitate reversibility and network disassembly (reviewed in [12–14,16,18]). As has been described for other ubiquitin binding types of NZFs [39], mutation of the threonine and phenylalanine residues within the $_{13}TF_{14}/\Phi_{25}$ motif of the Rybp protein (TF-AA mutant) completely abrogates binding to ubiquitin (Fig. 1B, upper graph, diamonds). Finally, the structure of the Npl4 NZF domain in complex with ubiquitin indicates that it binds at the essential I44A surface of ubiquitin [40]. In agreement with this, wildtype Rybp protein is unable to bind an I44A ubiquitin mutant (Fig. 1B, lower graph, squares).

Having shown that Rybp can bind to free ubiquitin in solution via its NZF, we next asked whether Rybp also could bind to ubiquitinated proteins (Fig. 1C). Whole cell lysates were prepared from HeLa cells that were either non-transfected or transfected with Myc-tagged ubiquitin, and equal amounts of these lysates were applied to columns of immobilized GST-Rybp or GST-Rybp(TF-AA). Bound proteins were resolved by SDS–PAGE followed by Western blotting with an anti-ubiquitin antibody. As shown in Fig. 1C, GST-Rybp is capable of associating with a smear of ubiquitinated proteins (top panel, lane 1) which intensifies when the proteins are harvested from cells transfected with ubiquitin (top panel, lane 2). This smear is not apparent when the NZF mutant of Rybp (GST-Rybp(TF-AA) is employed (Fig. 1C, top panel, lanes 4–5) nor when the GST fusions are incubated with binding buffer alone (Fig. 1C, top panel, lanes 3 and 6). Taken together, these findings suggest that Rybp can bind to ubiquitin and to ubiquitinated proteins in vitro in an NZF-dependent manner.

Next we asked whether Rybp also is capable of binding to ubiquitinated proteins in vivo (Fig. 2A). HeLa cells were transfected with FLAG-tagged Rybp with or without Myc-tagged ubiquitin, and lysates were immunoprecipitated with anti-FLAG antibody and blotted with anti-Myc tag antibody. As shown in Fig. 2A, lane 2, the co-introduction of Rybp and ubiquitin yielded a smear of ubiquitinated proteins in the anti-FLAG immunoprecipitates that was undetectable when the transfected proteins were introduced individually (Fig. 2A, lanes 1 and 4). Notably, this smear is significantly diminished in the FLAG immunoprecipitates from Rybp-$(TF\text{-}AA)^{FLAG}$ + ubiquitin transfected cells (Fig. 2A, lane 3). To ensure that equivalent levels of the wildtype and mutant versions of Rybp were immunoprecipitated in these studies, the FLAG immunoprecipitates also were probed with an anti-Rybp antibody (Fig. 2B, top panel). In addition to confirming this (see bands marked by open circle in Fig. 2B, top panel), the anti-Rybp antibody also detected a higher molecular weight band in the immunoprecipitates from wildtype Rybp + ubiquitin transfected cells only (see band marked by asterisk in Fig. 2B, top panel, lane 2). Given the apparent molecular weight of this band (~48 kDa), we suspected that it could represent $Rybp^{FLAG}$ modified by one moiety of Myc-tagged ubiquitin. This was verified when the FLAG immunoprecipitations were performed under high stringency buffer conditions, and the immunoprecipitates were blotted with the anti-Myc tag antibody (Fig. 2B, middle panel, lane 2, starred band). Accordingly, it appears that Rybp can be monoubiquitinated. Interestingly, modification of Rybp also depends upon its having an intact NZF, since the TF-AA mutant does not appear to be monoubiquitinated (Fig. 2B, all panels, lane 3) (note that the TF-AA mutation does not involve any lysine residues that could be ubiquitination targets). This "coupling" between ubiquitin binding by and ubiquitination of a UBD-containing protein, with both processes dependent upon the UBD, has been reported previously (reviewed in [12–14,16]; also see [42] for coupling reported for NZF UBDs). Recently, it has been shown that (mono)ubiquitination of UBD-containing proteins can serve a negative regulatory role, by promoting intramolecular interactions and preventing constitutive recognition of free ubiquitin and of ubiquitinated targets [43]. However, monoubiquitination of UBD-containing proteins may have other functional consequences upon these ubiquitin binding proteins as well, including mediating signal amplification (reviewed in [12–14,16]). The ramifications of Rybp's modification by ubiquitin remain to be determined.

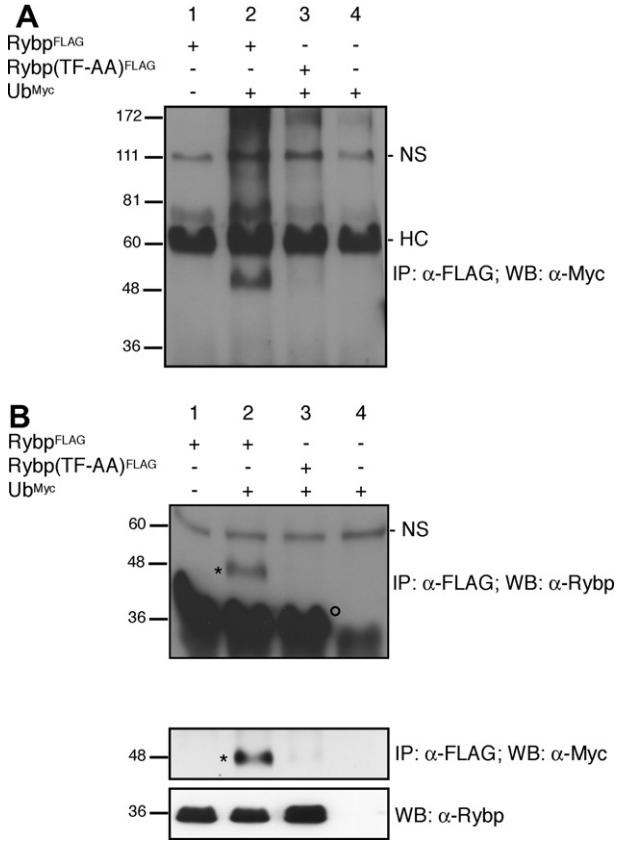

Fig. 2. Rybp binds to ubiquitinated proteins, and is itself monoubiquitinated, in vivo. Western blotting analysis of FLAG immunoprecipitates of HeLa cells transfected with the indicated plasmids, and probed with the anti-Myc antibody in (A) or the anti-Rybp antibody in (B), upper panel. In the middle panel in (B), FLAG-immunoprecipitations were performed on protein lysates prepared under high stringency conditions, and probed with the anti-Myc antibody. These lysates were also probed with the anti-Rybp antibody (B, lower panel) to confirm equal representation of the transfected Rybp proteins. The starred bands in (B) represent the monoubiquitinated form of Rybp; the bands marked by open circles in the upper panel of (B) represent unmodified, Flag-tagged Rybp or Rybp(TF-AA). Molecular weight is shown in kDa on the left. IP = immunoprecipitated; WB = Western blotting; HC = heavy chain; NS = non-specific.

3.2. *Effects of disrupting Rybp's ability to bind to and to be modified by ubiquitin*

Having shown that the Rybp(TF-AA) mutant does not appear to recognize ubiquitin or to be ubiquitinated, we next asked whether loss of these abilities affect some of the known properties of Rybp (Figs. 3 and 4). First we assessed whether this mutation affects Rybp's ability to interact with the Polycomb group (PcG) proteins Ring1A and Ring1B (also see [19]). FLAG-tagged versions of wildtype or mutant Rybp were tested for interaction with HA-tagged Ring1A or Myc-tagged Ring1B after transfection into HeLa cells. Wildtype Rybp and the Rybp(TF-AA) mutant were capable of immunoprecipitating comparable amounts of Ring1A (Fig. 3, top panel, lanes 2 and 4) or Ring1B (Fig. 3, top panel, lanes 7 and 9). FLAG-tagged Rybp and FLAG-tagged Rybp(TF-AA) appear to be expressed at similar levels (Fig. 3, lower panels), and the levels of introduced Ring1A or Ring1B were not changed in the various transfection points (Fig. 3, middle panels). These results suggest that Rybp's interaction with Ring1A/B is likely upstream or independent of Rybp's ability to recognize ubiquitinated proteins via an intact NZF.

Next we assessed whether disruption of Rybp's NZF UBD alters its subcellular distribution pattern. Wildtype Rybp or its TF-AA mutant was expressed in U2OS cells as YFP fusions, and subcellular localization was determined by fluorescent microscopy. As shown in Fig. 4A, YFP-Rybp assumed a characteristic punctate pattern in the nucleoplasm, and was also seen in larger subnuclear structures (Fig. 4A, left panel). These structures also were observed, albeit to a minor extent, when a CFP-Ring1B fusion protein was introduced by itself (Fig. 4A, right panel), and likely represent PcG bodies which have been reported previously to be sites for Ring1B and Rybp co-localization (see [19]). YFP-Rybp(TF-AA) was nuclearly

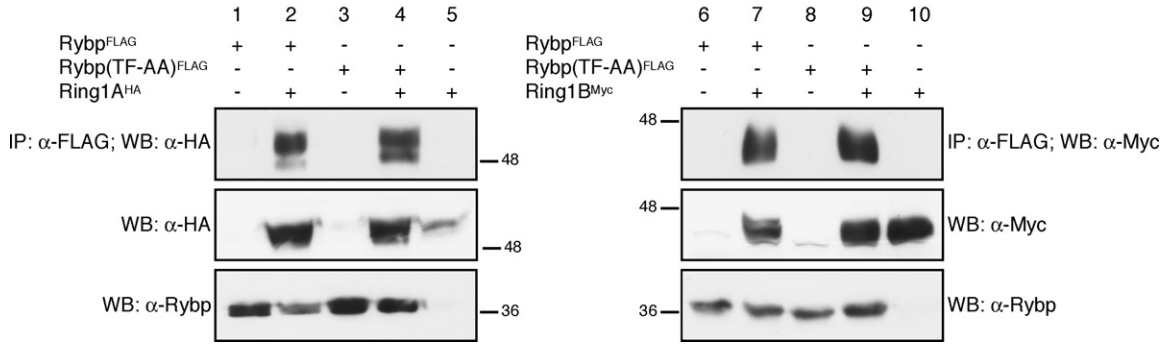

Fig. 3. Rybp NZF-UBD mutations do not alter its interaction with the PcG proteins Ring1A and Ring1B. Western blotting analyses of FLAG-immunoprecipitates from HeLa cells transfected with the indicated plasmids. Both Rybp and Rybp(TF-AA) interact similarly with Ring1A (as detected by the anti-HA antibody, upper left panel, compare lanes 2 and 4) and Ring1B (detected by the anti-Myc antibody, upper right panel, lanes 7 and 9). All constructs express at comparable levels, as indicated by Western blotting analyses on total protein lysates using antibodies directed against the HA tag (left, middle panel), against the Myc tag (right, middle panel), and against Rybp (bottom panels). Note that with slightly longer exposure times, endogenous Rybp is detectable in the Western panels probed with the anti-Rybp antibody. Molecular weight is shown in kDa. IP = immunoprecipitated; WB = Western blotting.

localized like YFP-Rybp, although its localization pattern comprised fewer discrete dots, and instead more aggregates therein (Fig. 4A, middle panel). Notably, when YFP-Rybp was co-introduced with CFP-Ring1B, the two proteins were found to co-localize to more prominent PcG bodies (Fig. 4B, upper row; see also [19]). In contrast, the YFP-Rybp(TF-AA) mutant and CFP-Ring1B did not colocalize, and PcG bodies were not seen (Fig. 4B, lower row). Western blotting analysis conducted upon lysates from the cells transfected with the various fusion proteins showed that YFP-Rybp and YFP-Rybp(TF-AA) were expressed at levels comparable to those of endogenous Rybp (see Fig. 4C, top panels), and that CFP-Ring1B levels were comparable to those of endogenous Ring1B (see Fig. 4C, bottom panels). Together, these immunofluorescence studies suggest that Rybp and Ring1B can co-localize in PcG bodies when expressed together in U2OS cells, and their overlapping pattern (and possibly the integrity of PcG bodies) depends upon Rybp carrying an intact NZF domain. How the localization and co-localization differences observed for the Rybp(TF-AA) mutant may relate to its inability to bind to ubiquitinated proteins remains to be determined.

3.3. Rybp ubiquitination is enhanced by the ubiquitin E3 ligase Ring1B

Having determined that Rybp is ubiquitinated and having reconfirmed that it interacts with and can be found together with Ring1B, we next asked whether Ring1B, a known ubiquitin E3 ligase [28–31,44], could enhance the monoubiquitination status of Rybp. For these assays (Fig. 5A), HeLa cells were transfected with FLAG-tagged Rybp with or without ubiquitin or Ring1B, and lysates were immunoprecipitated with the anti-FLAG antibody and blotted with the anti-Rybp antibody. The addition of ubiquitin to Rybp transfections results in the appearance of the expected ~48 kDa band that represents Rybp with a single ubiquitin moiety attached (Fig. 5A, starred band in lane 2). As reported previously for other ubiquitin binding proteins (reviewed in [14]), only a minor fraction of Rybp is ubiquitinated. However, when Ring1B is also added, this fraction is clearly increased (Fig. 5A, compare lanes 3 to 2 for starred band). Supporting the fact that this band indeed represents monoubiquitinated Rybp, when different tags were fused to the introduced ubiquitin moiety (*e.g.*, the Myc versus the 6X-HIS tag), the band migrated in accordance with the difference in molecular weight of the tags themselves (Fig. 5A, compare arrowed band in lane 4 to starred band in 3). Of note, we also tested how the Rybp(TF-AA) ubiquitin-binding mutant, which appears to have lost the ability to be monoubiquitinated (Figs. 2B and 5B), responds to the presence of Ring1B. Surprisingly, this mutant can become monoubiquitinated in the presence of over-expressed Ring1B (Fig. 5B, compare lane 3 starred band to lane 2). Taken together, our results suggest that Ring1B enhances the ubiquitination status of Rybp, and (at least in the engineered TF-AA mutant) can also function to uncouple Rybp's ability to be post-translationally modified from its ability to recognize ubiquitin. Whether Ring1B is a bona fide E3 ligase for Rybp, as it is for other proteins including histone H2A [28–31], needs to be clarified under more physiological settings.

3.4. Ubiquitinated histone H2A is a potential target of Rybp's sensing mechanism

Next we tested whether Rybp is able to colocalize with Ring1B and Ring1B's substrate, ubiquitinated histone H2A, on the inactive X chromosome in trophoblast stem (TS) cells [28,29]. Immunofluorescence analysis was performed on TS cells using antibodies directed against Ring1B, Rybp, and uH2A (Fig. 6A). Rybp was found to co-localize with Ring1B in almost 100% of the cells examined, and both proteins were concentrated on nuclear structures representing inactive X chromosomes (Fig. 6A, upper row). Notably, extensive co-localization of Rybp and uH2A at the inactive X was also observed (Fig. 6A, bottom row).

To pursue the uH2A-Rybp connection further, we asked whether ubiquitinated histone H2A could be a target for Rybp's ubiquitin binding activity. Nucleosomal histones were prepared and tested for interaction with recombinant GST-Rybp, GST-Rybp(TF-AA), or GST alone as a negative control (Fig. 6B and data not shown). Western blotting analysis showed that GST-Rybp can bind both to unmodified histone H2A and to ubiquitinated histone H2A (Fig. 6B, lane 2, upper and lower panels). In contrast, the interaction of GST-Rybp(TF-AA) with unmodified histone H2A was significantly

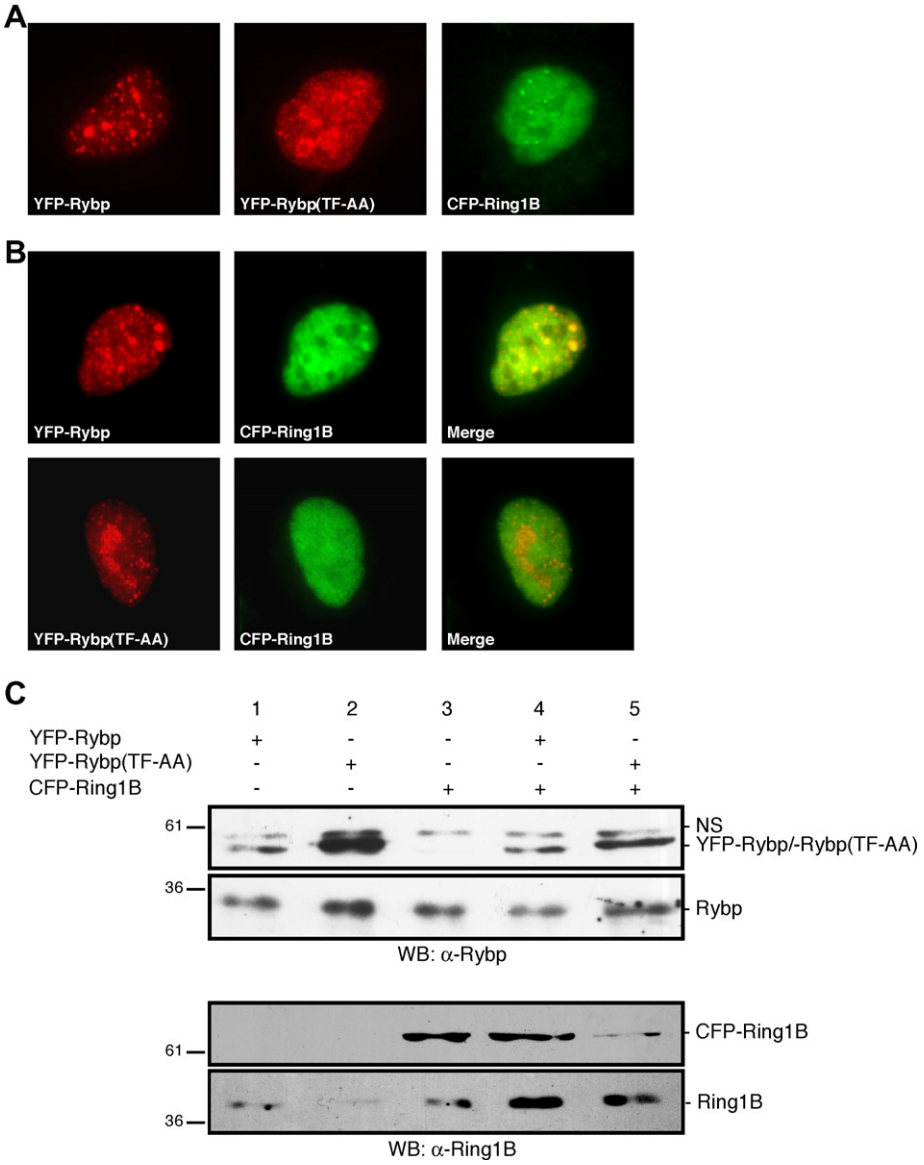

Fig. 4. Rybp NZF-UBD mutations affect its sub-nuclear localization and its colocalization with Ring1B. (A) and (B) representative fluorescent microscopy images of transfected U2OS cells, the majority of which exhibited the patterns shown here. In (A), YFP-Rybp, YFP-Rybp(TF-AA), or CFP-Ring1B was introduced alone. In (B), YFP-Rybp or YFP-Rybp(TF-AA) was co-introduced with CFP-Ring1B. (C) Western blotting analyses of protein lysates from U2OS cells transfected with the indicated fusion constructs, using antibodies directed against Rybp (upper panels) or Ring1B (lower panels). Endogenous (non-tagged) protein levels are shown in separate panels. Molecular weight is shown in kDa on the left. WB = Western blotting; NS = non-specific.

diminished, and that with ubiquitinated histone H2A was undetectable (Fig. 6B, lane 3, upper and lower panels). No binding was seen for pull-downs performed with GST alone (data not shown). Taken together, these findings suggest that ubiquitinated histone H2A is a potential target of Rybp's ubiquitin-binding activity. The fact that Rybp can also bind to unmodified histone H2A is not surprising given that ubiquitin binding proteins are believed to establish additional protein contacts (aside from with the ubiquitin moiety) to increase the affinity and specificity for their targets [14].

3.5. Implications

By establishing here that Rybp's ubiquitination status can be enhanced by Ring1B (Fig. 5), and that Rybp may influence Ring1B's subcellular localization in an NZF-dependent manner (Fig. 4), we have provided potential molecular explanations for the reported association of Rybp with PcG proteins. Our finding that one possible target of Rybp's NZF is ubiquitinated histone H2A (Fig. 6B) supports a possibly more active role for Rybp in PcG-mediated transcriptional repression. In particular, histone H2A ubiquitination has been shown to be mediated, at least in part, by Ring1B, and this modification has been linked to PcG-mediated gene silencing including the X inactivation process [28–31]. Our data from the trophoblast stem cell studies, demonstrating colocalization on the inactive X of Rybp, Ring1B, and ubH2A (Fig. 6A), suggest that Rybp may also be participating in gene silencing of the X chromosome. Finally, we cannot rule out the possibility

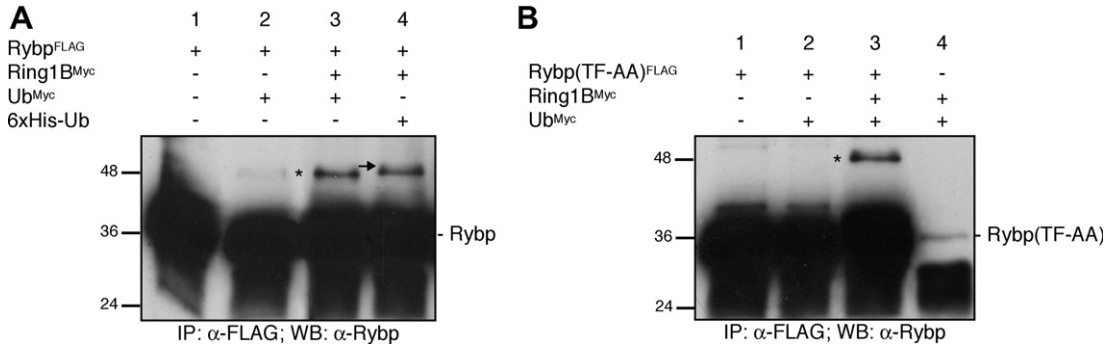

Fig. 5. The ubiquitin E3 ligase Ring1B enhances Rybp monoubiquitination. Western blotting analysis of FLAG immunoprecipitates of HeLa cells transfected with the indicated plasmids, including wildtype Rybp in (A) and the Rybp(TF-AA) mutant in (B). Monoubiquitinated Rybp forms are marked by asterisks, and unmodified introduced Rybp forms are indicated. The monoubiquitinated Rybp band migrates more slowly (arrowed band in lane 4) when 6XHis-Ub is introduced instead of Ub-myc. Ub = ubiquitin; WB = Western Blot; IP = immunoprecipitated.

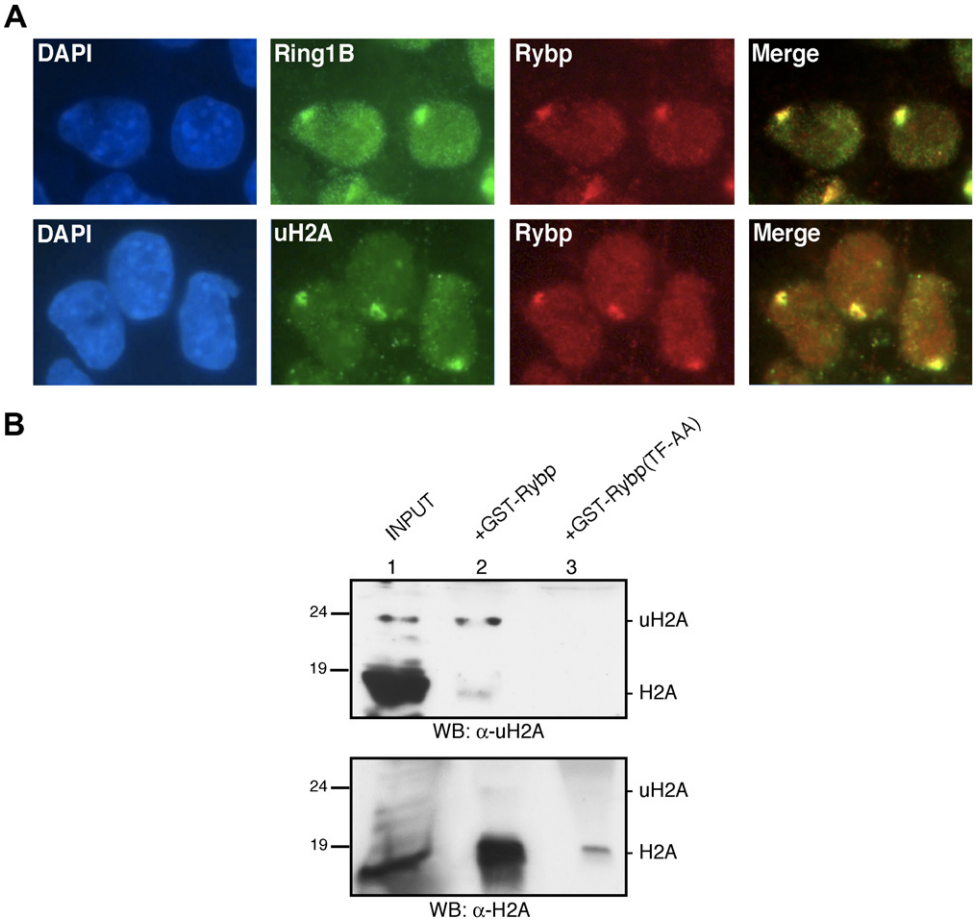

Fig. 6. Rybp and uH2A interact in vitro and co-localize in trophoblast stem cells. (A) Immunofluorescence analysis of trophoblast stem (TS) cells using antibodies directed against Ring1B (green, upper row), ubiquitinated histone H2A (green, lower row) and Rybp (red). Nuclei of TS cells were stained using DAPI (blue). Co-localization of Ring1B and Rybp or of uH2A and Rybp is seen as a yellow signal in the merge panels. This colocalization is especially apparent on the inactive X chromosome (bright spots). 63× magnification, with oil. (B) Western blotting analysis of pull-down experiments using GST-Rybp (lane 2) or GST-Rybp(TF-AA) (lane 3) incubated with nuclear pellet fractions from HeLa cells, and probed with antibodies against ubiquityl-histone H2A (upper panel) or histone H2A (lower panel). Ubiquitinated (uH2A) and non-modified (H2A) histone H2A are indicated on the right. A fraction of the input proteins is also shown (lane 1, 1/2 input was loaded in the upper panel, and 1/4 input was loaded in the lower panel). Molecular weights are indicated in kDa on the left. WB = Western blot.

that Rybp may play additional roles relating to the PcG complex, such as promoting protein–protein interactions between PRC1 members, modifying the activities of these proteins, or targeting the complex to other factors or to specific subcellular

compartments (the latter is also suggested from the results of Fig. 4).

A pressing future objective is to identify the spectrum of cellular ubiquitinated proteins that are recognized by Rybp and its NZF UBD; the collection of known Rybp interacting proteins and Rybp-containing protein complexes provides likely candidates for this (see [19,45–50]). If the modification status of these candidate interacting proteins is indeed "read" by Rybp, then this would help to explain why Rybp is able to interact with a wide variety of proteins localized to distinct subcellular compartments including Polycomb group proteins [19,48], DNA binding transcription factors [19,45,46,48], and apoptotic proteins [47,49]. Beyond the identification of target proteins, the precise functional consequences of Rybp's binding to these modified proteins remain to be elucidated.

Acknowledgements: The authors thank Drs. Jack Lenz, Ian Willis, Jon Warner, Richard Stanley, Liang Zhu, as well as members of the Schreiber-Agus laboratory, for stimulating discussions and critical reading of the manuscript. We are indebted to Dr. Jennifer Blanck for her contributions in the early phases of this study, Dr. Dmitry Fyodorov for helpful advice, and Dr. Cristina Montagna and the Albert Einstein Genome Imaging Facility for assistance in microscopy. Data in this paper are from a thesis submitted by RA in partial fulfillment of the requirements of the Degree of Doctor of Philosophy in the Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University. This work was supported in part by Public Health Service Grants from the NIH: CA92558 (N.S.A.), CA71540 (V.J.B.), and AI51174 (W.I.S.). S.L.A. was supported in part by IRG 77-003-23 from the American Cancer Society. Funds from the New York Speaker's Fund for Biomedical Research (N.S.A.), and support from the Albert Einstein Cancer Center (N.S.A.) are acknowledged as well.

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