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Evidence for distinct functions for human DNA repair factors hHR23A and hHR23B

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Abstract **Rad23 proteins bind ubiquitinated substrates and the proteasome, consistent with an important role in protein degradation. Although human Rad23 proteins (hHR23A and hHR23B) have redundant roles in DNA repair, we determined they formed distinct interactions with proteasomes and multiubiquitinated proteins, but similar binding to Ataxin-3. Threonine-79 contributed to the weak proteasome-binding property of hHR23A, and its conversion to proline (T79P), which is the residue present in hHR23B, increased proteasome interaction. We also determined that hHR23A and hHR23B could be co-purified with unique proteolytic and stress-responsive factors from human breast cancer tissues, indicating that they have unique functions in vivo.**

Keywords: hHR23; Proteasome; UBA; Ubiquitin; UbL; Ataxin-3

1. Introduction

Rad23 contains an amino-terminal ubiquitin-like (UbL) domain [1] that can bind the proteasome [2], and two ubiquitin-associated (UBA) domains [3,4] that bind Ub [5,6], and multiubiquitinated substrates [7,8]. Rad23/proteasome binding is required for efficient nucleotide excision repair (NER) [1,9], which is the primary mechanism for removing UV-induced DNA lesions. Although hHR23A and hHR23B are redundant in a reconstituted NER reaction [10], the requirement for two Rad23 homologs in metazoans is unresolved. NER is not required for viability, although the loss of both proteins causes embryonic lethality in mice [11]. This finding indicates that Rad23 has additional functions that are required for cell survival [8,12].

To identify functional differences between hHR23A and hHR23B we examined their interactions with the proteasome, multiubiquitinated (multi-Ub) proteins, and other cellular factors. We found that both proteins formed equivalent interaction with Ataxin-3 [13], a causative agent in the neurodegenerative disease spinocerebellar ataxia-3 (SCA3). However, hHR23A and hHR23B formed distinct interactions with the proteasome and multi-Ub proteins. A single amino acid residue in hHR23A (threonine-79) was associated with its reduced interaction with the proteasome. Conversion of threonine-79 to proline, which is the residue present in hHR23B, significantly increased hHR23A/proteasome binding, without affecting its association with ubiquitinated proteins, or Ataxin-3. We identified unique binding partners for hHR23A and hHR23B in primary breast cancer tissue, supporting our hypothesis that human Rad23 proteins have certain non-overlapping cellular functions, despite similar roles in NER.

2. Materials and methods

2.1. Plasmids and strains

The UbL domains from human hHR23A (1–79) and hHR23B (1–78), and yeast Rad23 (1–77) and Dsk2 (1–76) were cloned into pCBGST1 [2]. All the constructs were generated using polymerase chain reaction (PCR), and cloned using appropriate DNA restriction sites. Expression from pCBGST1 is regulated by the P_{CUP1} promoter. Therefore, expression was initiated by the addition of 100 μM $CuSO_4$ to the growth medium. Cells were pelleted (after 4 h growth at 30 °C), resuspended in buffer containing 1% Triton-X 100 and a cocktail of protease inhibitors, and lysed by mechanical agitation using 450 μ glass beads and a bead-disruptor. Constructs for expression in *E. coli* were generated by PCR, and cloned into pGEX-2TK (Amersham-Bioscience). Mutations in the UbL domains were generated by PCR using DNA oligonucleotides. In all cases, multiple isolates were subjected to DNA sequencing to confirm the authenticity of the mutation (IDT Integrated Technologies).

2.2. Protein extracts and purification

GST-UbL proteins were purified from yeast, as described [2]. GST-UbL proteins were immobilized on glutathione-Sepharose, and interacting factors were released in SDS-containing buffer, separated in SDS–PAGE, and transferred to nitrocellulose. The filters were stained with Ponceau S prior to immunoblotting. The purification of GST-fusion proteins from *E. coli* was described [14].

2.3. Cell lines and tissue extracts

Cultured human cell lines (ATCC) were grown at 37 °C with 5% CO_2 and collected when growth approached 90% confluence. Cell pellets and primary human tissue specimens were suspended in Buffer A, lysed by sonication, and extracts prepared as described [15]. One milligram of extract was applied to the GST-UbL matrices, incubated for 4 h at 4 °C with gentle mixing. The beads were washed three times with Buffer A, suspended in SDS-containing buffer and the bound proteins resolved by SDS–PAGE.

2.4. Proteasome activity measurement

The chymotrypsin-like proteasome activity was measured with 40 μM substrate (N-Suc-LLVY-AMC, Boston Biochem) [15], using a Turner-700 fluorometer. All measurements were conducted in triplicate, and multiple independent extracts were characterized.

2.5. Immunological methods

Antibodies against proteasome subunits (BioMol International, LP), eEF1A (Upstate Biotechnology, NY), ubiquitin (Sigma Chemical Co., St. Louis, MO), vimentin (Lab Vision Corp., Fremont, CA), E1

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(Biomol International, LP), and Isopeptidase T (Abgent, Inc), were diluted as recommended by the manufacturers. Antibodies against Ataxin-3 (and yeast Rpt1), were generated at Pocono Rabbits (PA). The normal Ataxin-3 protein, containing 20 polyglutamine repeats (Ataxin-3^{Q20}), was expressed in *E. coli* as a GST-fusion protein in the vector pGEX-2TK. Expression of the GST-Ataxin3 fusion protein was induced by the addition of IPTG to the growth medium. The protein was purified on GST-Sepharose. Ataxin-3 was released from the beads following treatment with thrombin, and thrombin was selectively removed from the supernatant by incubation with benzamidine-Sepharose (Pharmacia). Ataxin-3 (lacking GST), was concentrated by filtration, and used as an antigen for the preparation of antibodies. Immunoblots were subjected to enhanced chemifluorescence and the X-ray films quantified using Kodak 1D densitometry software (Rochester, NY). Multiple exposures were examined.

2.6. Mass spectrometry

Protein bands were detected with Coomassie blue, excised, and identified by mass spectrometry at the Center for Advanced Proteomics Research-New Jersey Medical School. The number of positive identifications for several putative targets is indicated below in parenthesis. Isopeptidase T (11 peptides); Ubiquitin activating enzyme E1 (6 peptides); Translation elongation factor 2 (18 peptides); Translation elongation factor 1a (9 peptides); Heat shock cognate 71 kDa (4 peptides); BRCA1 (4 peptides); BRCA2 (2 peptides); Vimentin (60 peptides); mKIAA0467 (3 peptides).

3. Results

3.1. UbL domain from hHR23B, but not hHR23A, binds yeast proteasome

UbL domains from yeast Rad23 (residues 1–78) and Dsk2 (1–78), as well as human hHR23A (1–79) and hHR23B (1–78) were fused to glutathione *S*-transferase (GST), and expressed in yeast. Protein extracts were incubated with glutathione-Sepharose to isolate the fusion proteins, and the co-purification of proteasomes was examined. We confirmed a strong interaction between the proteasome and the UbL domain from yeast Rad23 (Fig. 1, lane 1). As expected, no interaction was observed with GST, or ubiquitin (lane 3), since mono-Ub does not show appreciable affinity for the proteasome [16]. The UbL domain of yeast Dsk2 showed weak interaction, consistent with previous findings [17]. Despite significant sequence similarity (~75%) between human UbL-hHR23A and UbL-hHR23B (hereafter termed UbL-A and UbL-B), only the latter bound the yeast proteasome (lane 6). The difference in this interaction with yeast proteasomes suggested that they might have unique functions in protein degradation. Therefore, we sought to confirm these observations in human cell extracts.

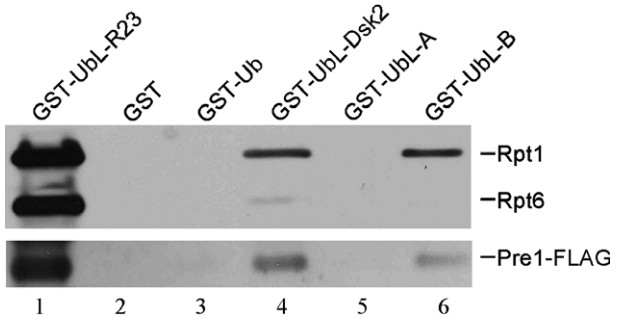

Fig. 1. Human UbL-A and UbL-B show differential interaction with yeast proteasomes. The interaction between yeast proteasomes and GST fusions encoding ubiquitin (Ub), and UbL domains from Rad23 (R23) Dsk2, hHR23A (UbL-A) and hHR23B (UbL-B) was determined, using antibodies against proteasome subunits Rpt1, Rpt6 and Pre1-FLAG.

3.2. UbL domains in hHR23A and hHR23B display unique interactions with the proteasome in mammalian cell extracts

Protein extracts from MCF10A, Hut78, and CHO cells were applied to GST-UbL-A and GST-UbL-B. Multiple cell lines were examined to avoid any cell-type specific effects. Ponceau S staining showed equal amounts of GST-UbL-A and GST-UbL-B were present on the affinity beads (upper panel in Fig. 2A). However, we detected ~3-fold higher levels of α-proteasome subunits with UbL-B (mean 2.94 ∓ 1.01; $P < 0.001$). High levels of 19S subunits Rpt1 and Rpn2 were also detected with UbL-B (data not shown; but see Fig. 3C) and, as expected, the chymotryptic activity associated with GST-UbL-B was higher (Fig. 2B). Similar findings were observed in ZR-75-1 breast cancer cells (data not shown), confirming that these binding differences between UbL-A and UbL-B are not cell-type specific. Addition of epoxomicin completely inhibited the chymotryptic activity, verifying the specificity of the proteasome assay (data not shown). The results obtained from 14 independent proteasome activity measurements were adjusted to the activity purified with UbL-A. Collectively, these results showed that the dissimilar proteasome-binding properties of UbL-A and -B are intrinsic properties of these domains, and is likely to have functional significance.

3.3. Full-length hHR23 proteins also display unique proteasome-binding properties

A previous study suggested that UBA and UbL domains in Rad23 might form an intramolecular interaction [17], which could affect Rad23/proteasome binding by restricting the availability of the UbL domain. To determine if full-length hHR23 proteins also displayed distinct proteasome-binding properties we immobilized GST-tagged hHR23A and hHR23B (that were purified from *E. coli*), and incubated with Hut78 protein extracts. Proteasome activity measurements from 10 independent assays were averaged and plotted (mean 2.39 ∓ 0.98; $P < 0.001$). We detected significantly higher levels of proteasome subunits, and proteasome activity, in association with hHR23B (Fig. 2C and D), consistent with the results obtained with UbL-B using the same extracts (Fig. 2A).

3.4. Distinct pattern of ubiquitinated species bind hHR23A and hHR23B

The UBA domain is a small, independently folded structure of ~40 amino acid residues that is present in a large number of proteins. A UBA domain in human Rad23 was first reported to bind the Vpr protein that is encoded by HIV-1. However, subsequent studies reported that UBA domains could interact with multi-Ub chains, and multiubiquitinated proteins (via the multi-Ub chain). Rad23 proteins contain two UBA domains, and the amino-terminal UBA1 domain typically forms a stronger interaction with multi-Ub chains [5,6]. However, both domains are required for maximum binding to ubiquitinated proteins [8]. Intriguingly, only the carboxy terminal UBA2 domain has been shown to bind other proteins, such as Vpr, p300 and MPG, suggesting that the two UBA sequences are functionally distinct.

We examined the interaction between multi-Ub proteins and full-length hHR23 proteins, as well as isolated UBA domains.

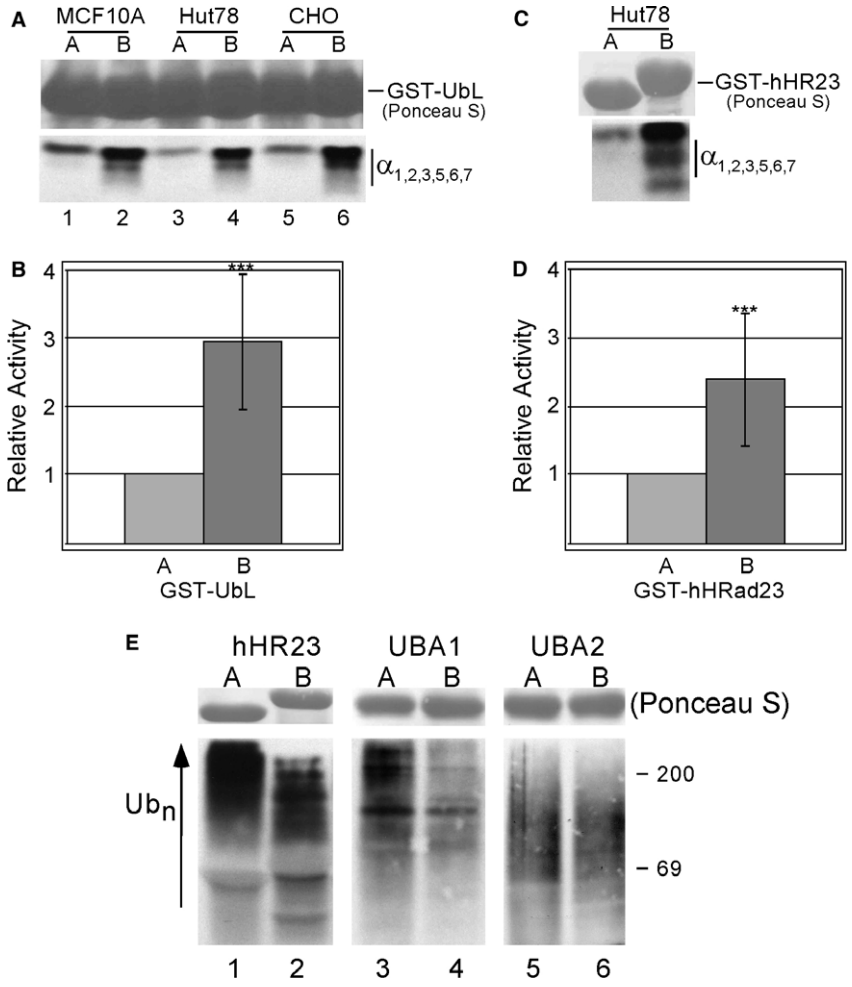

Fig. 2. Human Rad23 proteins show differential binding to proteasomes in mammalian extracts. (A) Equal amount GST-UbL-A and GST-UbL-B were incubated with extracts prepared from MCF10A, Hut78 and CHO cells. (B) Proteasome activity associated with GST-UbL-A beads was determined, and standardized to 1. Relative activity associated with GST-UbL-B is shown (*, $P < 0.001$). (C) The interaction between full-length GST-hHR23A/B and proteasome alpha subunits in Hut78 cell extracts is shown. (D) Relative proteasome activity associated with hHR23A/B was quantified and plotted (*, $P < 0.001$). (E) GST-tagged hHR23A/B, and their UBA1 domains, were incubated with Hut78 extracts and interaction with multi-Ub protein was determined by immunoblotting. Both hHR23A and UBA1-A formed >twofold increased interaction with multi-Ub proteins, than hHR23B and UBA1-B.

Immunoblotting with anti-ubiquitin antibodies revealed dissimilar patterns of interaction with hHR23A and hHR23B (Fig. 2E). Both hHR23A and UBA1-A (lanes 1 and 3) showed >2-fold increased binding to high molecular weight multi-Ub proteins, compared to hHR23B and UBA1-B (lanes 2 and 4). Furthermore, the multi-Ub species bound to hHR23A displayed a noticeably higher molecular weight distribution than the species bound to hHR23B. The significance of this difference is presently unclear.

3.5. UbL forms independent interactions with the proteasome and ataxin-3

Mutation of lysine-7 in the UbL domain of yeast Rad23 abolished interaction with the proteasome [18]. We generated a similar mutation in human UbL-A and UbL-B (Fig. 3A), and examined their interactions with the proteasome. Purified GST-UbL mutant proteins (Fig. 3B; UbL-A^{K8A} and UbL-B^{K6A}) were incubated with Hut78 cell extracts. Consistent with previous results, mutation of the residue corresponding to lysine-7 in yeast Rad23 abolished proteasome binding by both human UbL-A and UbL-B (Fig. 3C; lanes 6 and 7), and resulted in dramatically reduced co-purification of proteasome activity (Fig. 3D, lanes 6 and 7). The different proteasome-binding properties of the un-mutated UbL-A and UbL-B domains were also confirmed (Fig. 3D; compare lanes 2 and 3). As expected, lower levels of Rpn2, Rpt1 (Fig. 3C; lane 2), and alpha subunits (data not show) were purified with UbL-A.

To demonstrate that the reduced proteasome-binding by UbL-A was not caused by a structural defect, we examined its interaction with native Ataxin-3 present in the extract, using the same immunoblots. Ataxin-3 can bind the UbL domains in both human Rad23 proteins [13], and might encode a proteasome-associated factor that binds multiubiquitinated proteins [19]. The immunoblot shown in Fig. 3B was incubated with antibodies against Ataxin-3 and equivalent binding was observed for both UbL-A and UbL-B, indicating that the UbL domains were not structurally defective. These results indicate that distinct residues in UbL contribute to binding Ataxin-3 and the proteasome. The equivalent binding to Ataxin-3

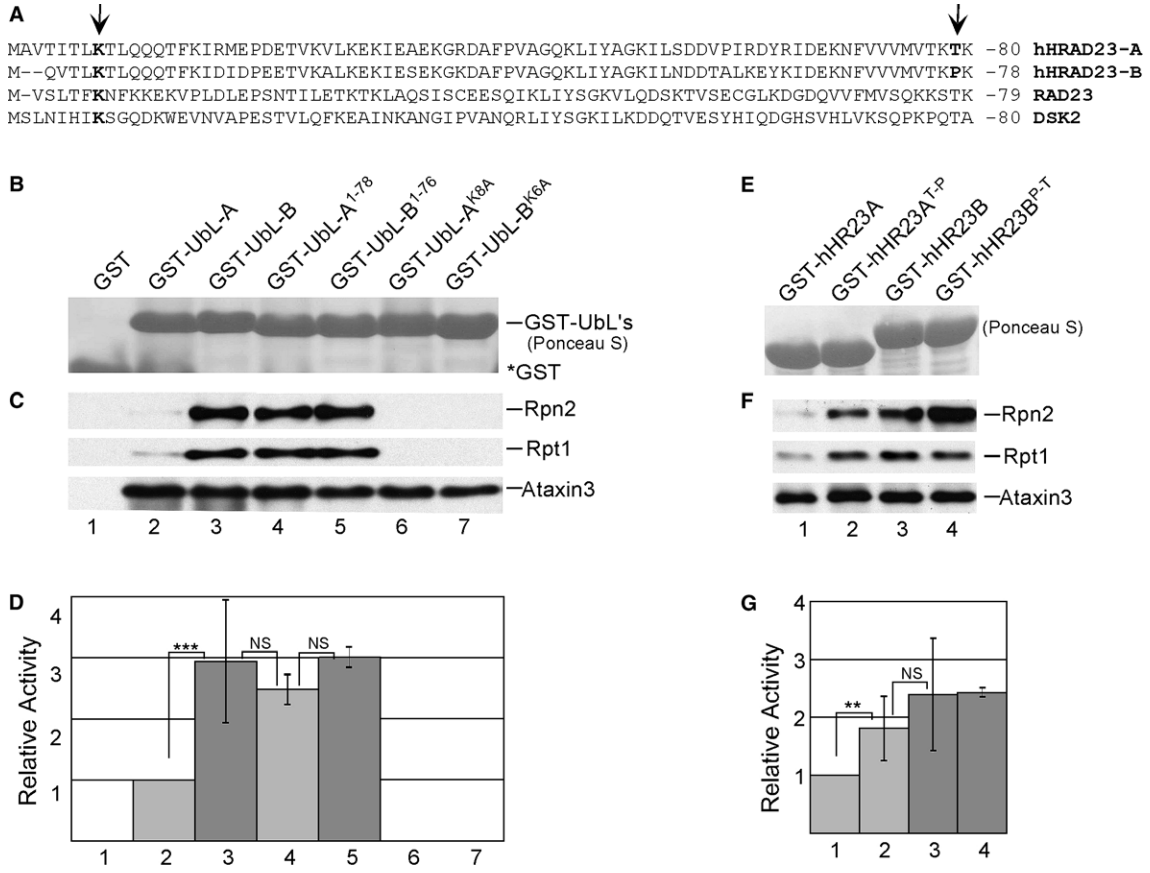

Fig. 3. Conversion of threonine-79 to proline-79 in hHR23A increased proteasome binding. (A) The amino acid sequence of the UbL domains is shown. Residues that were mutated (K7A, T79P and P77T) are indicated by the arrow. (B,E) GST fusion proteins were purified from *E. coli* and Ponceau S staining revealed purity, and equality of loading. (C) The affinity beads shown in B were incubated with 293 cell lysates, and the bound proteins were resolved in SDS–PAGE. An immunoblot was incubated with antibodies against proteins shown on the right. (D) Proteasome activity associated with the purified GST-fusion proteins shown in C, was determined from five independent experiments. The value associated with GST-UbL-A was adjusted to 1, and the relative activity detected with other GST-UbL's is shown (line 3, 4, 5). No proteasome binding or activity was detected with UbL^{K7A} (C and D, lanes 6 and 7). Removal of threonine-79 in UbL-A (1–78) increased proteasome binding and activity, to a level comparable with UbL-B (panels C and D; compare lanes 4 with 2; $P < 0.001$, and lane 3; $P > 0.05$). (F). The affinity beads shown in E were incubated with 293 cell lysates, and the bound proteins characterized by immunoblotting, using antibodies indicated on the right. (G). Proteasome activity for the reactions shown in F, were generated from eight measurements, and the activity associated with hHR23A was adjusted to 1 (lane 1). The levels bound to $hHR23A^{T79P}$ increased to hHR23B levels (compare lane 2 with 1; $P < 0.01$, and lane 3 with 2; $P > 0.05$), while the activity associated with $hHR23B^{P77T}$ was unchanged (lane 4, compare to lane 3).

strongly supports our hypothesis that the distinct proteasome binding properties of UbL-A and UbL-B are biologically significant. Furthermore, the inhibition of proteasome binding by the lysine mutation (similar to lysine-7 in yeast Rad23), also indicates that this mutation does not alter structure, since interaction with Ataxin-3 was unaffected.

We also examined Ataxin-3 interaction with full length hHR23A and hHR23B (Fig. 3E and F; compare lanes 1 and 3), and confirmed that GST-hHR23A and GST-hHR23B displayed differential binding to the proteasome (Fig. 3F; lanes 1 and 3). Consequently, purified hHR23A was associated with reduced proteasome activity (Fig. 3G). Similar to the results with the purified UbL domains, hHR23A and hHR23B interaction with Ataxin-3 was indistinguishable (Fig. 3F; lanes 1 and 3). These results demonstrate that the biochemical properties of the isolated UbL domains are recapitulated in the intact proteins, and suggest that the distinct binding properties conferred by the UbL-A and UbL-B domains are physiologically relevant.

3.6. Threonine-79 in UbL-A is associated with the reduced proteasome interaction

The amino acid sequences of UbL-A and UbL-B are highly conserved, although they display dissimilar proteasome-binding properties. An obvious difference between UbL-A and -B is the presence of additional residues at the amino-terminus in hHR23A (Fig. 3A). To determine if these residues inhibited interaction with the proteasome we generated a UbL-A derivative that lacked the additional residues (Ala^2 and Val^3). Conversion of these residues to alanine did not alter UbL-A/proteasome binding (data not shown). However, the cloning design of the GST-UbL proteins generated a sequence that terminated after 72-**VVVMVTK***T*-79 in UbL-A $^{1–79}$, and 72-**VVVMVTK***PK*-78 in UbL-B $^{1–78}$. To investigate if the different carboxy-termini affected proteasome-interaction we prepared constructs that terminated with the same residue (VVVMVTK; $UbL\text{-}A^{1–78}$ and $UbL\text{-}B^{1–76}$). Removal of threonine-79 from UbL-A increased $UbL\text{-}A^{1–78}$/proteasome binding (Fig. 3C; compare lanes 2 and 4). The associated chymotryptic activity

was determined from 5 independent measurements and also showed significant increase (mean increase 2.5 ∓ 0.25; $P < 0.001$). In contrast, proteasome-interaction by UbL-B^{1-76} was unchanged, and remained high (Fig. 3C; compare lanes 3 and 5). The chymotryptic activity purified with UbL-A^{1-78} was lower than UbL-B^{1-76}, although the difference was not significant ($P > 0.05$). As expected, UbL-A^{1-78} and UbL-B^{1-76} formed equivalent interaction with Ataxin-3 (Fig. 3C), demonstrating that Ataxin-3 and proteasome binding are separate properties.

We converted threonine-79 in full-length hHR23A to proline (T79P), which is the residue present in hHR23B (see arrow in Fig. 3A). Higher amounts of proteasome subunits (Rpn2 and Rpn1) were co-purified with GST-hHR23AP^{T79P} (Fig. 3F; lane 2), while the interaction with Ataxin-3 was unaffected. We measured chymotryptic activity that was purified with GST-hHR23A^{T79P}, GST-hHR23A and GST-hHR23B (Fig. 3G), and detected significantly higher levels with hHR23AP^{T79P}, compared to native hHR23A (Fig. 3G; compare lane 2 to lane 1; $P < 0.01$). The activity associated with hHR23AP^{T79P} did not increase to the levels detected with GST-hHR23B, although the difference was not significant (lanes 2 and 3; $P > 0.05$). Thus, a single amino acid residue that is present between the downstream sequences of hHR23A and UbL-A significantly affected proteasome binding. Intriguingly, a reciprocal substitution in hHR23B, in which Pro-77 was converted to Thr-77 (GST-hHR23B^{P77T}), did not reduce proteasome binding and chymotryptic activity to the levels observed with hHR23A (Fig. 3F and G; lane 4), suggesting that the negative effect of Thr-79 in hHR23A, may involve other residues in UbL-A. We also note that altering hHR23-A/proteasome interaction had no effect on its association with multi-Ub proteins (data not shown), consistent with our previous finding that Rad23 interactions with the proteasome and multi-Ub proteins are separable functions [8].

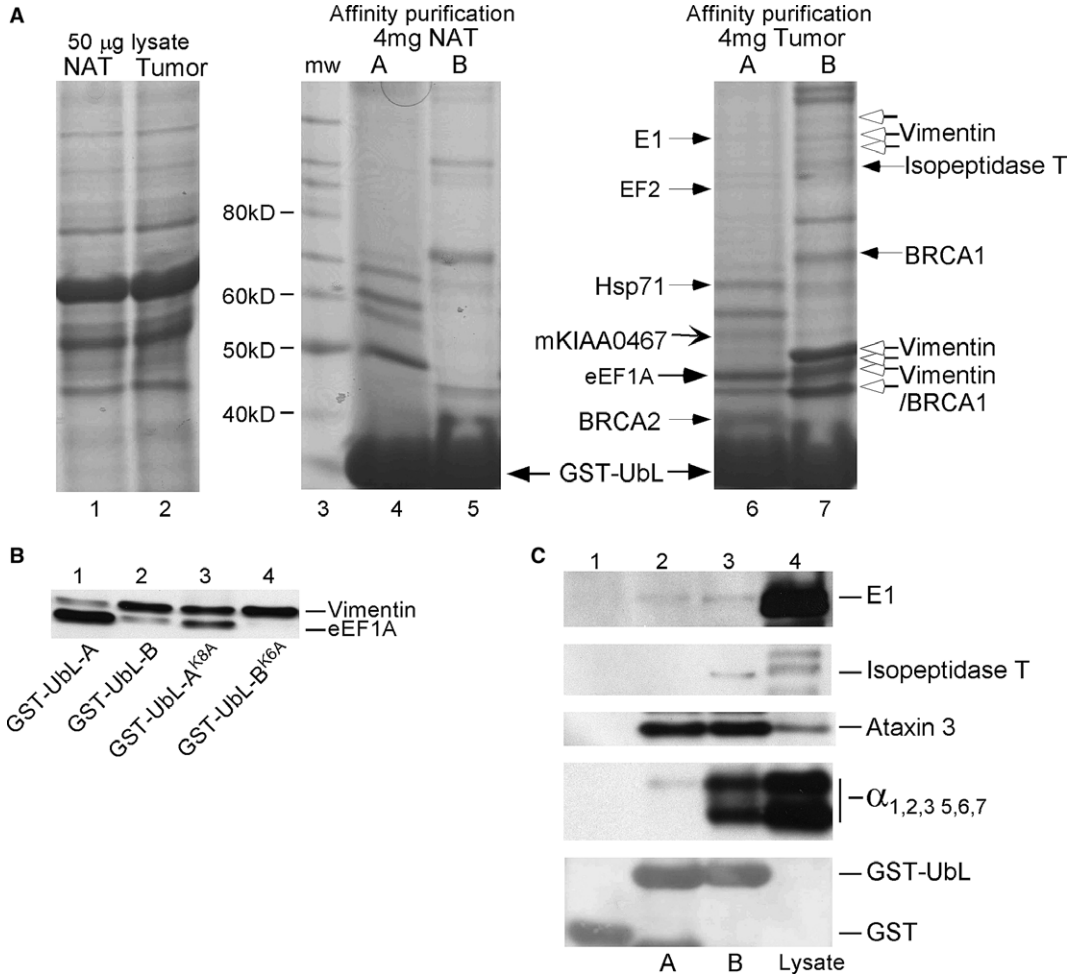

Fig. 4. Identification of novel UbL interactions in human primary breast cancer tissues. GST-UbL-A and GST-UbL-B were purified from *E. coli* and incubated with four milligrams of tissue extract prepared from human breast cancer and normal adjacent tissue (NAT). (A) Untreated protein extracts are shown in the left panel. GST-UbL proteins were incubated with tissue extracts and the profile of interacting proteins from normal tissue (NAT) is shown in the middle panel. Similarly, protein species that bound the UbL domains from breast cancer extracts is shown in the right panel. Specific protein bands were identified by mass spectrometry (see right panel). Proteins indicated on the left were detected primarily with UbL-A, while proteins named on the right were isolated with UbL-B. (B) Proteins in breast cancer tissue extracts that bound GST-UbL-A and GST-UbL-B were characterized with antibodies against vimentin and eEF1A. (C) Similarly, breast cancer extracts were applied to GST, GST-UbL-A and GST-UbL-B. The bound proteins were released in SDS-containing buffer, resolved by SDS–PAGE, and an immunoblot probed with antibodies against ubiquitin-activating enzyme E1, isopeptidase T, Ataxin-3, α-subunits of the 20S proteasome, and GST.

3.7. Proteomic approach reveals differences between hHR23A and hHR23B

To further characterize the biochemical properties of Rad23 we incubated UbL-A and UbL-B with protein extracts prepared from primary human breast cancer and normal adjacent tissues (NAT). The bound proteins were released, resolved in polyacrylamide gels, and stained with Coomassie blue. As expected, proteasome subunits were purified with the UbL domains (data not shown). However, we also purified novel cellular proteins from primary tissue extracts (Fig. 4A; lanes 6 and 7). Significantly, differences in these interactions were observed for UbL-A and UbL-B, in both cancer and in non-affected neighboring tissues that were derived from the same patient (Fig. 4A; lanes 4–7). Protein bands detected in breast cancer tissue extracts were excised and their identity revealed by mass spectrometry. A subset of candidate proteasome (or UbL) binding proteins is shown (Fig. 4A; lanes 6 and 7). UbL-A precipitated Ub-activating enzyme (E1), translation elongation factors (eEF1A and EF2), and fragments of BRCA2 (lane 6). Significant levels of the stress-inducible factor Hsp71 were also detected in association with UbL-A. In contrast, UbL-B was purified with Isopeptidase T, fragments of BRCA1, and various forms of the structural protein vimentin. BRCA1 is targeted for degradation by the Ub/proteasome pathway [20,21], and BRCA2 has been reported to be ubiquitinated [22]. Although both UbL-A and UbL-B can bind proteasomes, these findings suggest that the UbL domains might interact with compositionally distinct forms of the proteasome, which could reflect their different interactions with BRCA1 and BRCA2.

We used an immunoblotting approach to verify the proteomic analysis. GST-UbL fusion proteins were incubated with cell extracts to purify the proteasome and associated proteins. The bound proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and the immunoblots reacted with antibodies against vimentin. Consistent with the proteomic analysis, a predominant interaction was observed with UbL-B (Fig. 4B; lanes 1 and 2). In contrast, incubation with antibodies against translation elongation factor 1A (eEF1A), showed strong interaction with UbL-A, but significantly reduced binding to UbL-B (Fig. 4B; lanes 1 and 2). These results provide compelling support for the proteomic results in Fig. 4A, which shows differential interactions with the UbL domains. To further verify these results (Fig. 4A, lanes 6 and 7), we also investigated if other interactions (Fig. 4A; lanes 6 and 7), could also be confirmed immunologically. We incubated GST, GST-UbL-A and GST-UbL-B with breast cancer tissue extracts, and incubated an immunoblot with antibodies against E1, Isopeptidase T, Ataxin-3, and proteasome subunits (α1, 2, 3, 5, 6 and 7). Consistent with results described in Fig. 4B, we found that Isopeptidase T was only purified with GST-UbL-B (Panel C). In contrast, low levels of E1 were detected with both UbL domains, and high levels of Ataxin-3 were similarly detected with both domains. Despite this similarity, the interaction between the UbL domains and the proteasome was markedly different, as illustrated by the dissimilar co-purification of α-subunits of the 20S core proteasome particle (Fig. 4C). Taken together, these results, which examined the co-purification of E1, Isopeptidase T, Vimentin, eEF1A, Ataxin-3, and proteasome subunits and activity, provide compelling support for the hypothesis that human Rad23 proteins are functionally distinct. Our findings are also consistent with recent reports which showed that hHR23A can form an exclusive interaction with p53, while Rpn12 was preferentially purified with hHR23B [23]. Similarly, the HIV-1 Vpr protein might form a preferred interaction with hHR23A [24]. Collectively, these studies are consistent with our proteomic analysis, which indicates that human Rad23 proteins have unique functions.

4. Discussion

We describe distinct proteasome binding properties for human HR23A and -B proteins. Although hHR23A and hHR23B perform overlapping roles in NER, this DNA repair pathway is not required for survival. Therefore, the inviability of mice lacking both hHR23A and hHR23B [11], indicates that they have additional roles that contribute to cell survival. We determined that hHR23A and hHR23B form distinct interactions with the proteasome, and other cellular proteins. This unexpected result, demonstrates that hHR23 proteins are not redundant. These distinct interactions might contribute to the proteolytic functions proteins that are unrelated to their role in DNA repair.

A previous study showed that lysine-7 in the yeast Rad23 protein is required for efficient interaction with the proteasome. Based on the strong sequence conservation among Rad23 proteins sequences, we constructed similar mutations in the UbL domains from human hHR23A and hHR23B. The GST-UbL-A^{K8A} and GST-UbL-B^{K6A} mutant derivatives were purified from *E. coli* and incubated with extracts prepared from Hut78 cells. In agreement with previous results, we found that neither mutant UbL domain could interact with the proteasome, attesting to the strong evolutionary conservation among this class of proteins. Significantly, however, the interaction between the mutant UbL domains and Ataxin-3 was unaffected, supporting the idea that distinct residues contribute to these interactions, and confirming that the mutant UbL domains are not structurally impaired. Furthermore, the expression of these proteins was similar to their wildtype counterparts, indicating that they are not more unstable or prone to aggregation or precipitation. The distinct proteasome-binding properties of UbL-A and UbL-B were associated with the presence of a threonine residue at the carboxy-terminus of the UbL-A domain (T79). We converted this residue to proline, which is present in full-length hHR23B, and found that the proteasome-binding by $hHR23A^{T79P}$ increased considerably. Although this mutant did not bind the proteasome as efficiently as hHR23B, the improved binding was clear. It is also significant that this mutation improved interaction with the proteasome in the context of the native protein because it is likely that the structural integrity of the protein domains were maintained. It is interesting, however, that conversion of the proline residue in hHR23B to threonine (which is present in hHR23A), did not reduce interaction with the proteasome. This difference might be due to additional intermolecular interactions between the UbL domain and other sequences in the protein. It could be significant in this regard that the presence of proline could affect flexibility and relative positioning of the UbL domain in the intact Rad23 protein.

A proteomic analysis revealed differences in the protein factors that interact with hHR23A and hHR23B. Proteins

purified with the UbL domains were identified by mass spectrometry (Fig. 4). We presume that most of these interacting factors are bound to the proteasome. As expected, proteasome subunits, stress–response proteins (Hsp70 and Hsp71) and elongation factors, were co-purified with both UbL-A and UbL-B in breast cancer protein extracts. However, UbL-A and UbL-B also formed unique interactions. BRCA1 fragments, VCP/p97 (data not shown), and various forms of vimentin were purified primarily with GST-UbL-B. VCP/p97 is a member of a family of AAA-type ATPases that can bind multi-Ub chains and the proteasome [25].

eEF1A performs an essential function in translation elongation, although numerous connections between protein synthesis and protein degradation have been reported [26,27]. In addition, we showed that yeast eEF1A performs an important role in the co-translational degradation of nascent, misfolded proteins [28]. We determined that human eEF1A was purified primarily with UbL-A (Fig. 4B).

In summary, we show that hHR23 proteins form distinct interactions with various cellular factors, including proteasomes, multiubiquitinated proteins, and stress-related factors (eEF1A and Hsp's). Although hHR23A and hHR23B have similar functions in DNA repair, they evidently also perform distinct functions in vivo. These findings suggest that a detailed understanding of human Rad23 function will require characterization of both proteins in cancer, DNA repair and growth control.

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