A Conserved Deubiquitinating Enzyme Uses Intrinsically Disordered Regions to Scaffold Multiple Protein-Interaction Sites

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*Running title: IDRs in Ubp10/USP36 mediate protein interactions

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Background: Many proteins that regulate ubiquitination possess intrinsically disordered regions (IDRs), but little is known about how IDRs function.

Results: The yeast deubiquitinating enzyme Ubp10 has extensive IDRs with multiple, small protein-interaction motifs and this topology is conserved in Ubp10’s human ortholog USP36.

Conclusion: Ubp10/USP36 IDRs function as protein-interaction scaffolds.

Significance: We uncovered evolutionarily conserved ways that IDRs direct protein function in ubiquitination pathways.

ABSTRACT

In the canonical view of protein function, it is generally accepted that the three-dimensional (3D) structure of a protein determines its function. However, the past decade has seen a dramatic growth in the identification of proteins with extensive intrinsically disordered regions (IDRs), which are conformationally plastic and do not appear to adopt single 3D structures. One current paradigm for IDR function is that disorder enables IDRs to adopt multiple conformations, expanding the ability of a protein to interact with a wide variety of disparate proteins. The capacity for many interactions is an important feature of proteins that occupy the hubs of protein networks, in particular protein-modifying enzymes that usually have a broad spectrum of substrates. One such protein modification is ubiquitination, where ubiquitin is attached to proteins through ubiquitin ligases (E3s) and removed through deubiquitinating enzymes (DUBs). Numerous proteomic studies have found that thousands of proteins are dynamically regulated by cycles of ubiquitination and deubiquitination. Thus, how these enzymes target their wide array of substrates is of considerable importance for understanding the function of the cell’s diverse ubiquitination networks. Here, we characterize a yeast DUB, Ubp10, that possesses IDRs flanking its catalytic protease domain. We show that Ubp10 possesses multiple, distinct binding modules within its IDRs that are necessary and sufficient for directing protein interactions important for Ubp10’s known roles in gene silencing and ribosome biogenesis. The human homolog of Ubp10, USP36, also has IDRs flanking its catalytic domain, and these IDRs similarly contain binding modules important for protein interactions. This work highlights the significant protein-interaction scaffolding abilities of IDRs in the regulation of dynamic protein ubiquitination.
distinct conformations. This flexibility enables IDRs to interact with multiple proteins or interface with a single protein through distinct points of interaction (3,4,6,7). Studies on proteins with extensive IDRs have revealed them to be chief ‘hubs’ within different cellular networks (8-11). As such, understanding how IDRs use multiple motifs to regulate a cellular protein network is critical to understanding cell physiology.

One major class of cellular network hubs comprises the enzymes that mediate protein ubiquitination. Ubiquitination is a post-translational modification that regulates protein stability, localization, activity, and interactions (12). Consequences of protein ubiquitination are exceptionally broad, and this posttranslational modification controls nearly every cellular process. The covalent attachment of ubiquitin to substrates is achieved by an enzymatic cascade wherein a ubiquitin ligase, or E3, is the final component responsible for substrate recognition (12). Ubiquitination is dynamic and can be removed by the action of deubiquitinating enzymes, or DUBs (13). In humans, hundreds of E3s and DUBs are responsible for regulating thousands of substrates at any given time (12,13), and thus must be able to target a diverse network of proteins in response to different signaling events. As such, it is not surprising that ~20% of E3s are predicted to contain extensive IDRs (14).

Recent work from our lab revealed that the yeast DUB Ubp10 interacts with a large network of proteins in the nucleolus to regulate ribosome biogenesis (15). Previous work also identified the human ortholog of Ubp10, USP36, as having considerable nucleolar functions (16,17). In a comparative study with the intrinsically disordered E3 San1, we found that Ubp10 is predicted to have extensive IDRs flanking its catalytic domain (18). Because yeast Ubp10 interacts with dozens of nuclear/nucleolar proteins, we hypothesize that Ubp10 uses distinct motifs within its IDRs to interact with these different proteins. Here, we reveal that such motifs do exist within yeast Ubp10 and human USP36. These highly conserved DUBs offer invaluable tools to understand how IDRs maintain key protein interactions across evolution, and evolve new protein interactions as needed.

**EXPERIMENTAL PROCEDURES**

**Yeast strains and plasmids**—Yeast strains and plasmids used in this study are listed in Table 1. Standard yeast growth media and yeast genetic methods were employed (19). Standard cloning methods were used to construct each plasmid. The relevant portion of each plasmid was sequenced to verify the fusion sequences. Plasmid sequences, oligonucleotide sequences, and cloning details will be provided upon request.

**Expression and purification of Ubp10 and Dhr2**—We used the vector GST-TEV-UBP10 (a gift from Erik Zimmerman and Ning Zheng, University of Washington) for expression of Ubp10. We created the vector GST-TEV-DHR2 for expression of Dhr2. The vectors were transformed into T7 express cells (NEB). Transformed cells were grown in Luria broth (LB) plus ampicillin (100µg/ml final concentration) to ~1.3x10⁵ cells/ml. Protein expression was induced by addition of 250µM IPTG and 100µM ZnCl₂ (final concentrations) for 16 hours at 16°C. Cells were harvested and lysed with a microfluidizer in a buffer containing 200mM NaCl, 20mM Tris-Cl pH 8.0, 1mM PMSF, 5mM DTT. Soluble extract was applied to a GST resin (GE healthcare). Ubp10 and Dhr2 were cleaved from the resin with recombinant TEV protease, then concentrated using an Amicon Ultracel concentrator (cutoff ≥50 kDa).

**Deubiquitination assay**—To assay in vitro deubiquitinating activity of purified Ubp10, we used a ubiquitin conjugated 7-amido-4-methylcoumarin florescence (Ub-AMC) assay (20). Ub-AMC Assay Buffer (50mM HEPES pH 7.5, 0.5mM EDTA, 1mM DTT, 100µg/ml ovalbumin) was aliquoted (100µl/well) to ½ white AreaPlate (Perkin Elmer) 96 well plate. Purified Ubp10 was added either directly to wells in triplicate, or pre-incubated with the irreversible DUB inhibitor N-ethylmaleimide (NEM, Sigma) for 5 minutes at room temperature and then added in triplicate. Ubiquitin-AMC (Boston Biochem) was added to each mixture, which was then incubated at 30°C for 40 minutes. Fluorescence was measured with excitation at 346nm and emission at 442nm.

**Histone H2B deubiquitination assay**—To assay deubiquitination of histone H2B in vitro, we prepared whole cell yeast lysates from a 50ml culture of cells that expressed FLAG-tagged histone H2B grown in rich media at 30°C to a density of 1.5x10⁷ cells/ml. Cells were harvested by centrifugation, then lysed in 1ml lysis buffer (200mM NaCl, 20mM Tris pH 8.0, 1mM PMSF, 10mM NEM) by vortexing with glass beads. The isolated cell lysate was combined with 800µl lysis buffer wash of the beads. The combined lysate and
washed by centrifugation for 5 minutes at 13,000 x g. Histone H2B was immunopurified by combining the clarified lysate with 30 µl anti-Flag M2 affinity resin (Sigma). After mixing 16 hours at 4°C, the resin was washed 3x with 1 ml lysis buffer, then resuspended in 30 µl deubiquitination assay buffer (100 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol). 10 µl of resin slurry was incubated with purified Ubp10 for 50 minutes at 37°C. Proteins were eluted in SUMEB (1% SDS, 8 M Urea, 10 mM MOPS, pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) by incubating for 10 minutes at 65°C, then resolved by SDS-PAGE. Western analysis using anti-FLAG antibodies revealed the extent of histone H2B deubiquitination.

Limited proteolysis—Proteolysis of purified Ubp10 or commercially obtained bovine serum albumin (BSA, Sigma) was carried out by trypsin digestion at 25°C in digestion buffer (50 mM NaCl and 15 mM Na2HPO4, pH 7.3). The enzyme to substrate ratio was 1:50. Proteolysis was stopped by addition of SUMEB, then incubation at 100°C for 10 minutes. Digestion was examined by resolving protein fragments by SDS-PAGE followed by silver staining of the gel.

Circular Dichroism—Far-UV Circular Dichroism (CD) spectra of purified Ubp10 or BSA were obtained on an Aviv 62DS CD spectrometer. Spectra were recorded at 25°C with a 1 mm optical cuvette from 260 to 190 nm. An average of three runs was obtained by sampling every 2 nm with a 30 second averaging time. Each protein was resuspended in CD buffer (50 mM NaCl, 15 mM Na2HPO4, pH 7.3). Protein concentrations ranged between 5 and 10 mM. The background CD buffer spectrum was subtracted from each protein spectrum.

Yeast 2-Hybrid (Y2H) assay—Y2H assays were performed as previously described (21). Yeast cells expressing fusions of the Gal4 binding domain (GBD) with Ubp10 or USP36 containing the indicated mutations and the appropriate protein fusions to the Gal4 activation domain (GAD) were spotted onto selective (synthetic media minus histidine) and nonselective media (synthetic media plus histidine) to assess interactions and spotting efficiency, respectively. All interaction tests were performed in triplicate using at least two independent isolates.

Western analyses—Lysates generated from in vivo cultures were generated as previously described (15). Cultures were grown to a cell density of ~0.7x10^7 cells/ml. Cells were lysed by 5 minutes of bead beating at 4°C in SUMEB with 10 mM PMSF. Proteins were resolved on 6% SDS-PAGE gels for Rpa190 or 10% SDS-PAGE gels for histone H2B, transferred to nitrocellulose, and visualized by Western analyses using anti-Rpa190 antibodies (generously provided by David Schneider, University of Alabama – Birmingham) or anti-FLAG antibodies (Sigma).

Ubiquitin affinity chromatography—Purification of ubiquitinated proteins from yeast was performed as previously described (15). Cells expressing 8His-ubiquitin were grown in 50 ml rich media to a density of ~0.7x10^7 cells/ml. Harvested cells were lysed by 20 minutes of bead beating at 4°C in 1 ml lysis buffer (8 M Urea, 0.05% SDS, 50 mM Tris pH 8.0, 1 mM PMSF, 10 mM NEM). Lysates were clarified by centrifugation, and incubated with 40 µl slurry of TALON resin (Novagen) overnight at 4°C. The resin was washed 3x with 1 ml lysis buffer (8 M Urea, 0.05% SDS, 50 mM Tris pH 8.0, 1 mM PMSF, 10 mM NEM). Ubiquitinated proteins were eluted from the column by addition of 40 µl SUMEB. Proteins in the lysate and eluate were resolved with 6% SDS-PAGE gels and examined by Western analyses.

Image processing—Growth plates and Western blots were scanned using an Epson Perfection V350 Photo scanner at 300 dpi. All images were processed with a Mac iMac or Pro computer (Apple) using Photoshop CS or CS4 (Adobe).

RESULTS

Ubp10 deubiquitinates several chromatin-associated proteins including histone H2B (22,23), the DNA clamp PCNA (24,25), and the largest subunit of RNA polymerase I Rpa190 (15). While Ubp10 is recruited to telomere proximal regions by interaction with the silencing protein Sir4 to deubiquitinate histone H2B (22,23), very little is understood about how Ubp10 is directed to deubiquitinate its other known substrates.

Ubp10 contains intrinsically disordered regions. DUBs typically possess a core catalytic domain essential for their deubiquitinating activity with variable length N- and C-terminal extensions that are thought to direct their function (26). Ubp10 has a large N-terminal region (residues 1-359), a DUB catalytic domain (residues 360-729), and a small C-terminal region (residues 730-792) (Fig. 1A). To gain insight into Ubp10’s function, we analyzed Ubp10’s domain composition and secondary structure characteristics using protein domain, secondary structure, and order/disorder
prediction algorithms. The N- and C-terminal regions of Ubp10 contain no identifiable protein domains. Using the secondary structure predictor JPredict (27), we found there was little secondary structure predicted for Ubp10 in its N- and C-terminal regions compared with its catalytic domain (Fig. 1B). Therefore, we used different order/disorder prediction algorithms to determine the overall extent of Ubp10 structural disorder. We found that Ubp10 was predicted to contain high intrinsic disorder (≥85%) within its N- and C-terminal regions (Fig. 1C). The top panel in Figure 1C is the disorder prediction for Ubp10 by PONDR, which predicts disorder by comparing the sample protein sequence against a “neural network” that was developed from curated disordered regions identified through missing electron densities in x-ray crystallography or from disordered regions characterized by nuclear magnetic resonance (NMR) structures (28). The middle panel is the disorder prediction from FoldIndex, which predicts disorder in local protein sequence by quantifying the sequence’s net mean charge and net hydrophobicity (29). The bottom panel is the disorder prediction from IUPred, which predicts disorder by analyzing pairwise energies between local protein sequences and predicting the likelihood they will form globular structures (30). Interestingly, 5 of the 15 other yeast DUBs (Ubp1, Ubp3, Ubp4, Ubp7, and Ubp13) also possess IDRs of at least 100 residues in length (Fig. 2), indicating that disorder is common among DUBs, as has been shown for E3s (14). However, the massive extent of Ubp10’s disorder is unique among the yeast DUBs.

To determine experimentally if Ubp10 is intrinsically disordered, we purified recombinant Ubp10 from E. coli. We confirmed the enzymatic activity of purified Ubp10 using an in vitro fluorescent DUB assay. Purified Ubp10 deubiquitinated ubiquitin-conjugated AMC, which is indicated by a significant increase in the fluorescence signal (Fig. 3A). The increase in fluorescence was inhibited by preincubation of Ubp10 with the irreversible DUB inhibitor N-ethylmaleimide (NEM). Purified Ubp10 was also capable of deubiquitinating histone H2B in vitro, and this could also be inhibited by preincubation of Ubp10 with NEM (Fig. 3B).

Having confirmed purified Ubp10 is functionally active, we next used circular dichroism to identify the α-helical, β-sheet, and random coil secondary structure content of Ubp10. Purified Ubp10’s spectrum (Fig. 3C; black line) contains minima at 204 nm and 218 nm, indicative of the known α-helical structure in its DUB catalytic domain ((26) and Fig. 1A). However, when compared to the highly structured α-helical protein BSA (Fig. 3C; gray line), the shift of Ubp10’s spectrum downwards and towards 200 nm is characteristic of a protein with a high degree of random coil (31).

We also used limited proteolysis to assess Ubp10’s intrinsic disorder. Limited proteolysis takes advantage of the greater number of exposed protease cleavage sites in IDRs versus highly structured regions (32). Purified Ubp10 or BSA were exposed to trypsin over a time course of 45 minutes. Ubp10 was rapidly degraded with almost no visible full-length protein after 2 minutes of exposure to trypsin (Fig. 3D). This is in stark contrast to the highly structured protein BSA, which maintained the full-length protein throughout the 45 minutes of trypsin digestion. Thus, CD and limited proteolysis confirmed the predictions that Ubp10 contains considerable intrinsic disorder.

Ubp10 uses binding modules within its N-terminal IDR to direct multiple protein interactions. We recently demonstrated that Ubp10 is enriched in the nucleolus where it interacts with multiple proteins involved in ribosome biogenesis (15). Little is known about how Ubp10 interacts with these proteins. The only known protein-protein interaction information about Ubp10 comes from its interaction with the telomere-silencing protein Sir4. An earlier study found that the deletion of residues 94-250 in Ubp10’s N-terminal IDR was sufficient to disrupt its interaction with Sir4 (22). Therefore, we thought it likely that regions within Ubp10’s IDRs might direct its interaction with ribosome biogenesis proteins.

To identify if Ubp10 contains discrete binding modules within its N-terminal IDR, we used ANCHOR, an algorithm that predicts binding regions within IDRs (33). ANCHOR operates on three principles: 1) a given residue is within disorder, 2) that residue is not predicted to make favorable contacts with other in cis local residues, and 3) that residue is able to make favorable contacts with an in trans interactor (33). The results from ANCHOR predicted twelve binding modules in Ubp10’s N-terminal and C-terminal IDRs, ranging in length from 9-38 residues. These regions are shown in Figure 4A, with the predicted modules of binding shown below the graph.
We next determined whether the predicted binding modules were required for Ubp10’s protein interactions. Previously, we developed a yeast 2-hybrid (Y2H) assay that was capable of querying Ubp10 interactions (15). Using the Y2H assay, we examined three representative proteins known to interact with Ubp10: the silencing protein Sir4 (22,34), the rRNA DEAD-box helicase Dhr2 (15), and the rRNA processing factor Utp22 (15). We made short deletions in Ubp10 to remove the predicted binding modules in the IDRs or the separating intervals. Full-length and each deletion mutant Ubp10 were probed for interaction with Dhr2, Sir4, and Utp22 using the Y2H assay (Fig. 4B). Strikingly, each protein bound a distinct module within the N-terminal IDR of Ubp10. Residues 2-27 in Ubp10 were required to interact with Dhr2, and loss of these residues did not affect the interaction with Sir4 or Utp22. Likewise, Ubp10 required residues 109-122 and 123-141 to interact with Sir4, and loss of these residues had no effect on interaction with Dhr2 and Utp22. Finally, Ubp10 required residues 171-208 to interact with Utp22, and loss of these residues had no effect on interaction with Dhr2 and Sir4. Thus, each of the three representative Ubp10 interactions is defined by its own unique binding module within Ubp10’s N-terminal IDR.

Having identified unique binding modules within Ubp10 required for each interaction, we wanted to determine whether these modules alone were sufficient for interaction with each protein. To test this, we probed for interactions in the Y2H assay using the Ubp10 regions spanning residues 2-27, 109-141, or 171-208 to see if those modules by themselves were capable of interacting with Dhr2, Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). Residues 2-27 of Ubp10 solely interacted with Dhr2. Residues 109-141 of Ubp10 solely interacted with Sir4, and Utp22, respectively. We found that regions of Ubp10 required to interact with each protein were also sufficient for that interaction (Fig. 4C). The intrinsic disorder of Ubp10 is unchanged in the presence of a binding partner. We established that Ubp10 contains small binding modules that are necessary and sufficient for interaction with its partner proteins. It is possible that IDRs adopt structures when in contact with binding partners (4,5). We therefore wanted to test whether the IDRs of Ubp10 changed when interacting with a binding partner. To test this, we performed limited proteolysis with Ubp10 alone versus Ubp10 pre-incubated with its binding partner Dhr2 (Fig 5). There was no significant change in the limited proteolysis digestion pattern with or without Dhr2 (Fig 5), indicating that Ubp10’s disorder is not drastically changed when interacting with a protein partner.

Human USP36 is a functionally conserved ortholog of yeast Ubp10. To date, Ubp10 has two predicted eukaryotic orthologs: human USP36 and Drosophila scrawny. USP36 has been shown to localize predominantly to the human nucleolus and regulate the stability of the largest subunit of telomere chromatin silencing.
human RNA polymerase I, RPA194 (16,17,37), which is similar to Ubp10’s predominant localization to the yeast nucleolus and regulation of Rpa190 (15). Importantly, Ubp10’s human ortholog USP36 shows increased expression in ovarian cancer cells (38), and overexpression rescues the growth deficit of cancer cells (16). These studies indicate a key cancer link for USP36/Ubp10. Drosophila scrany has been shown to regulate histone H2B ubiquitination and chromatin silencing during Drosophila growth and development (39), similar to Ubp10’s control of histone H2B ubiquitination and chromatin silencing in yeast (22,23). Because RNA polymerase I stability and histone H2B regulation are fundamental to eukaryotes, we wanted to determine if USP36 is a broadly conserved functional homolog of Ubp10.

Previously, we found that expression of USP36 in yeast from the endogenous UBP10 promoter restored deubiquitination of yeast Rpa190 in ubp10Δ cells (15). Here, we examined if this was due to the catalytic action of USP36 or a noncatalytic feature of USP36. We mutated the USP36 catalytic cysteine residue (position 137) to serine and found that the catalytically inactive USP36C137S did not complement the ubp10Δ allele for Rpa190 deubiquitination (Fig. 6A). In addition, we previously reported that yeast cells deleted for UBP10 have a slow-growth phenotype due to reduced levels of Rpa190 that result from increased ubiquitin-mediated degradation of Rpa190 (15). Consistent with its ability to rescue the deubiquitination of Rpa190, expression of catalytically active human USP36, but not inactive USP36C137S, rescued the slow growth of yeast ubp10Δ cells (Fig. 6B). Thus, the catalytic function of USP36 is conserved for regulation of RNA polymerase I.

Histone H2B deubiquitination is an important epigenetic control for chromatin silencing (22,23,39). Histone H2B ubiquitination is elevated in ubp10Δ cells (22,23). We therefore explored if expression of catalytically active USP36 could complement the loss of Ubp10 function for histone H2B deubiquitination. We found that expression of active USP36, but not inactive USP36C137S, in ubp10Δ yeast cells resulted in decreased ubiquitination of histone H2B (Fig. 6C), consistent with conservation of chromatin function for USP36/Ubp10. However, expression of catalytically active USP36 did not rescue the chromatin-silencing defect of ubp10Δ cells (Fig. 6D). This is not surprising because human cells do not have a homolog of Sir4 and use the divergent protein HP1 for chromatin silencing (40). This also explains why rescue of histone H2B deubiquitination by expression of active USP36 in ubp10Δ cells was partial (Fig. 6C). Although Ubp10 deubiquitinates histone H2B in silent chromatin regions, it also does so throughout the genome in active regions (22,41). Thus, while the silencing defect could not be rescued by expression of active USP36, the euchromatin function for histone H2B deubiquitination was rescued.

USP36 is predicted to be disordered. While USP36 and Ubp10 share 27% identity and 49% similarity in their catalytic DUB domains (39), there is little homology throughout the rest of the proteins. Similar to our analyses of Ubp10, we explored the predicted secondary structure content of the USP36 N- and C-terminal regions and found there was little secondary structure compared to its catalytic domain (Fig. 7A,B). When we queried the order/disorder of USP36 using PONDR, FoldIndex, and IUPred (Fig. 7C), USP36’s N- and C-terminal regions were predicted to contain ≥87% intrinsic disorder, which is strikingly similar to Ubp10’s ≥85% intrinsic disorder in its N- and C-terminal regions. Thus, USP36 also contains N- and C-terminal extensions that are predicted to be highly disordered. Interestingly, the organization of each USP36 extension was opposite to that of Ubp10, with USP36 containing a short N-terminal IDR and a long C-terminal IDR (Fig. 7A). Given the evolutionary distance between yeast and humans, it is possible that the lengths of each arm changed due to loss and/or gain of species-specific binding modules (such as a Sir4-binding region in yeast Ubp10). Altogether, the order/disorder predictions suggest that USP36 likely uses binding modules in its IDRs similar to Ubp10.

USP36 contains a conserved binding module for yeast Dhr2. Having established that USP36 shares conserved function and predicted intrinsic disorder with Ubp10, it seemed likely that USP36 would also contain conserved binding modules as well. We probed the protein structure of USP36 with ANCHOR and found that there were 17 predicted binding modules ranging from 10-51 residues in length (Fig. 8A). USP36 has known roles in ribosome biogenesis and nucleolar structure (16,17,37), so we hypothesized that it may interact with nucleolar interactors of Ubp10. We used the Y2H assay to examine interactions between human USP36 and yeast Dhr2, Utp22, and Sir4. We found that USP36 interacted with
Dhr2, though it did not interact with Utp22 or Sir4 (Fig. 8B). We expected a lack of interaction with Sir4 since human cells do not possess an ortholog of Sir4. We could not find a human ortholog of yeast Utp22 by sequence identity. Thus, the lack of interaction between human USP36 and yeast Utp22 is not unexpected. There is a human conserved ortholog of Dhr2, DHX33, that was found to interact with USP36 in a global proteomic study of DUB interactions in human cells (42). These results lend confidence to the conservation of interaction between USP36 and yeast Dhr2 in the Y2H.

To identify whether USP36 contained a small, unique binding module for Dhr2 similar to Ubp10, we created truncations of USP36 to identify which regions of USP36’s N- and C-terminal IDRs were required for interaction. As a first start, we deleted the entire N- or C-terminal IDRs of USP36. Only deletion of the N-terminal IDR disrupted the interaction with Dhr2. This region contains two predicted binding modules spanning residues 27-40 and 49-63. We found that deletion of residues 49-63 disrupted the interaction with Dhr2 (Fig. 8C).

Intriguingly, the USP36 Dhr2-binding module had a divergent amino acid sequence from the Ubp10 Dhr2-binding module (Fig. 8D). This was surprising and we wondered if there was a common feature between the two modules that transcends sequence. Binding modules within IDRs have been shown to take on secondary structure when binding protein interactors. In fact, the Ubp10 Dhr2-binding module has the potential to form an α-helix (Fig. 1B, and underlined sequence in Fig. 8D). We plotted the Ubp10 Dhr2-binding module sequence on an α-helical wheel model (Fig. 8E, left panel), and the predicted result was an amphipathic helix with a hydrophobic face. Similarly, plotting the USP36 Dhr2-binding module sequence on an α-helical wheel model revealed it too was predicted to have an amphipathic helix with a hydrophobic face (Fig. 8E, right panel). Thus, it appears that the Ubp10 and USP36 Dhr2-interacting modules share a similar biochemical capacity, which could take on α-helical structure when in contact with similar surfaces of Dhr2/DHX33. Altogether, our results demonstrate that Ubp10 and USP36 each contain a binding module for Dhr2. The sequences differ, but the predicted biochemical characteristics are similar.

**IDRs in Ubp10/USP36 mediate protein interactions**

**DISCUSSION**

Dynamic protein ubiquitination is a crucial signaling mechanism used by eukaryotic cells to control the myriad physiological processes essential for life. These include regulation of the cell cycle, control of metabolic pathway output, modulation of chromatin structure during DNA replication and transcription, and maintenance of protein quality. Because the number of substrates greatly exceeds the limited number of ubiquitin ligases (E3s) and deubiquitinating enzymes (DUBs), how these enzymes achieve their broad substrate targeting is of considerable interest to understanding the behavior of the different ubiquitination networks.

Intrinsic disorder grants proteins a structural plasticity that often facilitates interactions with multiple protein partners, so it is not surprising that IDRs are predicted to be common in ubiquitination pathway proteins (14). Limited previous studies have documented the functional use of intrinsic disorder in particular E3s such as San1 (21), Sic1 (43), BRCA1 (44), and HDM2/MDM2 (45). However, very little is known about the function of IDRs in DUBs. Our studies here are the first to explore how IDRs confer functionality in an evolutionarily conserved DUB.

IDRs can mediate interactions with multiple proteins and/or facilitate multiple interactions with a single protein using small linear sequence motifs within the IDRs (3,6,46). Here, we demonstrate that the yeast DUB Ubp10 contains multiple small linear motifs within its N-terminal IDR that each direct distinct protein interactions. Although our data suggest that each binding motif is unique for a particular protein interaction (Dhr2, Sir4, or Utp22), it is possible that each binding motif also has multiple targets within the cell. More Ubp10 interactions will need to be queried to determine if the unique binding modules within Ubp10’s IDRs have a one-to-one relationship with their targets or if they have a one-to-many relationship (6,46). It also remains to be determined whether the distinct IDRs of Ubp10 remain intrinsically disordered or adopt a localized structure upon binding its protein targets. We don’t think the latter is the case because Ubp10’s proteolytic vulnerability was unchanged when its binding partner Dhr2 was present in a limited proteolysis reaction.

Both Ubp10 and its human homolog USP36 share a similar topological profile in that they have extensive IDRs surrounding their DUB catalytic domain. Furthermore, both proteins are predicted to contain a series of small binding motifs within...
their IDRs. However, Ubp10 possesses a large N-terminal IDR whereas USP36 possesses a large C-terminal IDR. If we consider that the IDRs are modular, then the differing size and topology of the IDRs could have evolved by addition or subtraction of binding motifs that confer species-specific interactions. For example, Ubp10 acquired a motif that allows interaction with Sir4, which is a protein that is not present in humans. Furthermore, exon rearrangements could have also led to the different configurations of Ubp10 and USP36 whereby the exon encoding the catalytic domain shifted its position with the overall topology of the IDRs. Since IDRs lack structure, this shifting would not cause undue problems for the function of DUBs that use IDRs for binding partners or substrates.

Expression of USP36 complemented for loss of Ubp10 in yeast for a number of functions including deubiquitination of histone H2B and Rpa190. Thus, it is clear that conserved motifs exist in each DUB. This is exemplified by the fact that both DUBs interact with yeast Dhr2, an RNA helicase involved in ribosome biogenesis that has a well defined human ortholog, DHX33, shown to interact with USP36 (42). However, human USP36 did not interact with yeast Utp22, which is another key protein involved in ribosome biogenesis. This surprised us at first, until we searched the literature on the conservation of ribosome biogenesis factors. In our search, we found that most ribosome biogenesis factors are conserved between yeast and humans, but there are cases where there is no clear sequence ortholog (47). Because we could not identify a human ortholog for Utp22 by sequence searching, it is likely that the function for Utp22 may have diverged between yeast and humans. If so, USP36 would have lost the ability to interact with the functional analog in yeast.

Our results presented here demonstrate the powerful ability to utilize different predictive software in conjunction with molecular biology to identify the structural and functional capabilities of IDRs in dynamic ubiquitination pathways. In particular, we focused on intrinsic disorder in two evolutionarily conserved DUBs that appear drastically different by sequence identity and domain composition. Characterization of these proteins provides new models to understand how IDRs scaffold linear motifs to coordinate functional protein-protein interactions across large evolutionary distances.

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Author contributions: The overall study and experimental design were conceived by RGG, BJR, and MNL. Experiments for each figure were conducted by the noted people in the order of their experimental effort as follows: Figure 1–MNL, RGG; Figure 2–BJR, RGG; Figure 3–MNL, BJR; Figure 4–MNL, BJR; Figure 5–BJR; Figure 6–BJR; Figure 7–BJR, RGG; Figure 8–BJR. Primary writing of the manuscript was done by RGG and BJR. Revisions of the manuscript were done by RGG, BJR, and MNL. All authors reviewed the results and approved the final version of the manuscript.

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**FOOTNOTES**
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2 The abbreviations used are: IDR, intrinsically disordered region; DUB, deubiquitinating enzyme; AMC, 7-amido-4-methylcoumarin; NEM, N-ethylmaleimide; GST, glutathione S-transferase; TEV, tobacco etch virus; IPTG, isopropyl β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NIH, National Institutes of Health; NIA, National Institute on Aging; NIGMS, National Institute of General Medical Sciences

**FIGURE LEGENDS**

FIGURE 1. **Ubp10 is predicted to be disordered.** *A*, A schematic representing overall topology of Ubp10. *B*, Secondary structure prediction for Ubp10 using JPred (http://www.compbio.dundee.ac.uk/www-jpred/) (27). α-helices are denoted in black and β-sheets are denoted in gray. *C*, Disorder predictions of Ubp10. Top panel is PONDR (http://www.pondr.com/). Middle panel is FoldIndex (http://bip.weizmann.ac.il/fldbin/findex). Bottom panel is IUPred (http://iupred.enzim.hu/index.html). Predicted disordered regions are dark gray and predicted ordered regions are light gray. Ubp10’s catalytic domain is within 359-730.

FIGURE 2. **IUPred disorder prediction plots for yeast deubiquitinating enzymes (DUBs).** Each IUPred plot is labeled for its respective DUB (http://iupred.enzim.hu/index.html).

FIGURE 3. **Ubp10 is disordered.** *A*, Ub-AMC was incubated alone, with Ubp10, or with Ubp10 + 10mM NEM for 40 minutes. Total fluorescence for each condition was plotted as Relative Fluorescence Units, which was calculated by normalizing each condition to the fluorescent signal of Ub-AMC alone. *B*, Ubiquitinated histone H2B was purified from S. cerevisiae and incubated alone, with Ubp10, or with Ubp10 + 10mM NEM for 50 minutes. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and the Western blot was probed with anti-FLAG antibodies. *C*, Circular Dichroism (CD) spectra of Ubp10 (black) and BSA (gray) were recorded at 0.15 mg/ml for Ubp10 and BSA in 50 mM NaCl and 15 mM NaHPO₄, pH 7.3 at 25°C. *D*, Purified Ubp10 or BSA was incubated with trypsin for the indicated times. Proteins were separated by SDS-PAGE and visualized by silver stain. The locations of trypsin (*), Ubp10 (†), and BSA (††) are marked.

FIGURE 4. **Small linear regions in Ubp10’s N-terminal IDR are necessary and sufficient for binding Dhr2, Sir4 and Utp22.** *A*, ANCHOR prediction of binding sites in Ubp10 (http://anchor.enzim.hu/). Arrows above the graph indicate which binding region is required for each protein interaction. Bars below the graph indicate the residues of Ubp10 that comprise each predicted binding module. *B*, Yeast 2-hybrid (Y2H) interaction assays in cells expressing each predicted binding module (GBD)-Ubp10 deletion variant (left) and Gal4 activation domain (GAD) fusion (top). Cells were spotted onto synthetic media with or without histidine to measure spotting efficiency and the Y2H interaction, respectively. *C*, Cells
expressing full length GBD-Ubp10 or GBD-Ubp10 peptide sequence (left) and the indicated GAD fusion (top) were spotted onto synthetic media with or without histidine to measure spotting efficiency and the Y2H interaction, respectively. D, Yeast cells containing URA3 near telomere VII (22) and the indicated Ubp10 or Sir4 mutants (left) were spotted onto synthetic media with or without uracil to measure spotting efficiency and chromatin silencing.

FIGURE 5. Limited proteolysis of Ubp10 is unchanged in the presence of Dhr2. Purified Ubp10, Dhr2, or both were incubated with trypsin for the indicated times. Proteins were separated by SDS-PAGE and visualized by silver stain. The locations of Ubp10 (†) and Dhr2 (††) are marked.

FIGURE 6. Expression of USP36 in ubp10Δ yeast cells complements loss of Ubp10 function for Rpa190 and histone H2B deubiquitination. A, UBP10, ubp10Δ, ubp10Δ/UBP10, ubp10Δ/USP36, and ubp10Δ/USP36C137S cells expressing 8HIS-ubiquitin were lysed in 8M Urea, 0.05% SDS, 50mM Tris pH 8.0. Ubiquitinated proteins were affinity purified by metal affinity chromatography (15). Lysates and eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose, and Western blots probed with anti-Rpa190 antibodies. B, UBP10, ubp10Δ, ubp10Δ/UBP10, ubp10Δ/USP36, and ubp10Δ/USP36C137S cells were spotted onto rich media and grown to measure colony size. C, UBP10, ubp10Δ, ubp10Δ/UBP10, ubp10Δ/USP36, and ubp10Δ/USP36C137S cells were lysed in SUMEB, proteins separated by SDS-PAGE, transferred to nitrocellulose, and Western blots probed with anti-FLAG antibodies to detect histone H2B. Note that the yeast DUB gene UBP8 was also deleted in all strains to maximize our ability to see increases in histone H2B ubiquitination (22,23). D, UBP10, ubp10Δ, sir4Δ, ubp10/UBP10, ubp10Δ/USP36, and ubp10Δ/USP36C137S cells expressing URA3 near telomere VII (22) to measure chromatin silencing were spotted onto synthetic media with or without uracil. Spotting efficiency is measured by growth on synthetic media with uracil. Loss of chromatin silencing is measured by growth on synthetic media without uracil.

FIGURE 7. USP36 is predicted to be disordered. A, A schematic representing overall topology of USP36. B, Secondary structure prediction for USP36 using JPred (http://www.compbio.dundee.ac.uk/www-jpred/) (27). α-helices are denoted in black and β-sheets are denoted in gray. C, Disorder predictions of USP36. Top panel is PONDR (http://www.pondr.com/). Middle panel is FoldIndex (http://bip.weizmann.ac.il/fldbin/findex). Bottom panel is IUPred (http://iupred.enzim.hu/index.html). Predicted disordered regions are dark gray and predicted ordered regions are light gray. Ubp10’s catalytic domain is within 359-730.

FIGURE 8. Small linear region in USP36’s N-terminal IDR is necessary and sufficient for binding yeast Dhr2. A, ANCHOR prediction of binding sites in USP36 (http://anchor.enzim.hu/). Bars below the graph indicate the residues of USP36 associated with each binding module. B, Y2H interaction assays in cells expressing the indicated GBD-USP36 deletion (left) and GAD fusion (top) were spotted onto synthetic media with or without histidine to measure spotting efficiency and the Y2H interaction, respectively. C, Cells expressing full length GBD-USP36 or USP36 peptide sequence (left) and GAD fusion (top) were spotted onto synthetic media with or without histidine to measure spotting efficiency and the Y2H, respectively. D, Sequences of the Dhr2-interacting modules in Ubp10 and USP36. E, α-helical wheel mapping of the Dhr2-interacting modules in Ubp10 and USP36. The mappings were made using http://rzlab.ucr.edu/scripts/wheel/wheel.cgi. Hydrophobic residues are marked in black.
TABLE 1: Plasmids and Yeast strains used in this study

| Plasmid   | Alias     | Figure(s) | Plasmid description                  | Reference |
|-----------|-----------|-----------|--------------------------------------|-----------|
| pRG118    | pGBD-C1   | Figures 4 & 8 | P_{ADH1}-GBD (empty vector), TRP1 | (48)      |
| pRG1260   |           | Figure 4   | P_{ADH1}-GBD-UBP10(C371S), TRP1    | (18)      |
| pRG1332   |           | Figures 4 & 8 | P_{ADH1}-GAD-SIR4(612-1358), LEU2  | This study|
| pRG2490   |           | Figures 4 & 8 | P_{ADH1}-GAD-DHR2, LEU2           | This study|
| pRG2747   |           | Figures 3 & 5 | P_{COOL}-GST-TEV-UBP10          | This study|
| pRG3352   |           | Figures 4 & 8 | P_{ADH1}-GAD-UTP22, LEU2         | This study|
| pRG4201   |           | Figure 8   | P_{ADH1}-GBD-USP36, TRP1          | This study|
| pRG4232   |           | Figure 6   | P_{UPB10}-USP36(C131S)-3HSV, TRP1 | This study|
| pRG4233   |           | Figure 5   | P_{COOL}-GST-TEV-DHR2            | This study|

| Strain    | Figure(s) | Yeast strain description                  | Reference |
|-----------|-----------|------------------------------------------|-----------|
| PJ69-4A   | Figures 4 & 8 | MATa his3Δ200 ura3-52 trp1-901 leu2-3,122 | (48)      |
|           |           | met2::P_{GAL7}-lacZ lys2::P_{GAL7}-HIS3 ade2::P_{GAL7}-ADE2 gal4Δ gal80Δ |          |
| RGY658    | UCC6389   | Figure 6 | MATa ade2Δ::hisG met15Δ0 his3Δ200 ura3Δ0 trp1Δ63 lys2Δ0 leu2Δ0 HTA1-FLAG-HTB1 | (22)      |
| RGY659    | UCC6390   | Figure 6 | UCC6389 ubp10Δ::NatMX               | (22)      |
| RGY662    | UCC6393   | Figure 6 | UCC6389 ubp10Δ::NatMX ubp8Δ::KanMX  | (22)      |
| RGY799    | UCC6422   | Figure 4 | MATa ade2Δ::hisG met15Δ0 his3Δ200 ura3Δ0 trp1Δ63 lys2Δ0 leu2Δ0 TEL-VIL::P_{ADH1}-URA3 | (22)      |
| RGY800    | UCC6423   | Figure 4 | RGY799 ubp10Δ::NatMX                | (22)      |
| RGY801    | UCC6424   | Figure 4 | RGY799 sir4Δ::KanMX                 | (22)      |
| RGY4468   |           | Figure 6 | RGY662 ubp10Δ::NatMX::USP36         | This study|
| RGY4546   |           | Figure 6 | MATa met15Δ0 his3Δ0 ura3Δ0 leu2Δ0   | This study|
|           |           | rpal40Δ::8HIS-Rpl40a rps31::8H-rps31   |          |
|           |           | ubi4Δ::8H-ubi-8H-ubi-8H-ubi-8H-ubi-8H-ubi |          |
| RGY4728   |           | Figure 6 | RGY4546 ubp10Δ::NatMX               | This study|
| RGY4945   |           | Figure 6 | RGY4728 ubp10Δ::NatMX::USP36        | This study|
| RGY5453   |           | Figure 4 | RGY800 ubp10Δ::NatMX::ubp10-3HSV    | This study|
| RGY5454   |           | Figure 4 | RGY800 ubp10Δ::NatMX::ubp10(Δ109-133)-3HSV | This study|
| RGY5454   |           | Figure 4 | RGY800 ubp10Δ::NatMX::ubp10(Δ171-208)-3HSV | This study|
| RGY5487   |           | Figure 4 | RGY800 ubp10Δ::NatMX::ubp10(Δ2-27)-3HSV | This study|
| RGY5914   |           | Figure 6 | RGY800 ubp10Δ::NatMX::usp36(C131S)-3HSV | This study|
IDRs in Ubp10/USP36 mediate protein interactions

Figure 1
Figure 2

IDRs in Ubp10/USP36 mediate protein interactions
IDRs in Ubp10/USP36 mediate protein interactions

Figure 3
**Figure 4**

**A** IDRs in Ubp10/USP36 mediate protein interactions

**B**

|        | Dhr2 | Sir4 | Utp22 |
|--------|------|------|-------|
| no fusion |      |      |       |
| full length |      |      |       |
| Δ2-27   |      |      |       |
| Δ28-40  |      |      |       |
| Δ41-60  |      |      |       |
| Δ51-58  |      |      |       |
| Δ59-75  |      |      |       |
| Δ76-86  |      |      |       |
| Δ87-102 |      |      |       |
| Δ103-108|      |      |       |
| Δ109-122|      |      |       |
| Δ123-141|      |      |       |
| Δ142-160|      |      |       |
| Δ161-170|      |      |       |
| Δ171-208|      |      |       |
| Δ209-218|      |      |       |
| Δ219-251|      |      |       |
| Δ252-260|      |      |       |
| Δ261-288|      |      |       |
| Δ289-320|      |      |       |
| Δ321-335|      |      |       |
| Δ725-733|      |      |       |
| Δ734-770|      |      |       |
| Δ771-792|      |      |       |

**C**

**D**

|        | spotting efficiency | 2-hybrid interaction |
|--------|----------------------|-----------------------|
| no fusion |                      |                       |
| full length |                      |                       |
| 2-27    |                      |                       |
| 109-141 |                      |                       |
| 171-208 |                      |                       |

**Diagrams**

- Dhr2
- Sir4
- Utp22

**Silencing assay**

- parent
- ubp10 Δ
- sir4 Δ
- UBP10
- UBP10 Δ
- UBP10Δ
- UBP10ΔΔ
- UBP10ΔΔΔ
IDRs in Ubp10/USP36 mediate protein interactions
IDRs in Ubp10/USP36 mediate protein interactions
Figure 7

IDRs in Ubp10/USP36 mediate protein interactions
IDRs in Ubp10/USP36 mediate protein interactions

Figure 8
A Conserved Deubiquitinating Enzyme Uses Intrinsically Disordered Regions to Scaffold Multiple Protein-Interaction Sites
Benjamin J. Reed, Melissa N. Locke and Richard G. Gardner

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