Abstract

High levels of reactive oxidative species (ROS) induce oxidative stress in cells, disrupting many cellular processes. To mitigate the harmful effects of oxidative stress, cells engage in counteracting mechanisms by reprogramming gene expression and protein activity. Among these mechanisms, cells must regulate global translation to promote cellular viability during stress conditions. In yeast, cells utilize K63-linked polyubiquitin chains (K63ub) to modify ribosomes and aid in the control protein synthesis during stress. K63ub has been shown to heavily accumulate on ribosomes in response to oxidative stress, stalling translational elongation. However, little is known about the role and regulation of the important enzymes that govern this response. Although we have previously identified Ubp2 as a key deubiquitinating enzyme (DUB) involved in the accumulation of K63ub during stress, my objective was to determine whether and how the redox regulation of Ubp2 modulates stress-induced K63ub dynamics. Using western blot, cellular and activity assays, and molecular modeling, I showed that Ubp2, a cysteine-dependent DUB, is reversibly inhibited upon cellular treatment with H_2O_2, resulting in an accumulation of K63ub on ribosomes. Furthermore, I identified and characterized three repeated domains that are key regulators of Ubp2's activity during oxidative stress recovery. Overall, this study suggests a novel mechanism by which Ubp2 regulates K63ub dynamics in the translational oxidative stress response and establishes a basis for understanding the potential redox regulation of additional DUBs during stress. Understanding how Ubp2's structure governs its stress-induced regulation could elucidate a more comprehensive understanding of cellular resistance to oxidative stress, potentially informing treatment for various stress-related diseases.

INTRODUCTION

Eukaryotic cells must constantly detect and adapt to challenging conditions that affect cellular health. One such condition, known as oxidative stress, arises when the production and accumulation of reactive oxygen species (ROS) surpasses the biological system's ability to detoxify these reactive products [1]. ROS, such as hydrogen peroxide (H_2O_2), are normal byproducts of oxygen metabolism and play an important physiological role in many cellular processes (i.e., cell signaling, regulation of cellular functioning, protein phosphorylation, and transcription factor activation) [1, 2]. However, excessive amounts of ROS cause macromolecular damage by oxidizing important biomolecules such as lipids, nucleic acids, and proteins [3]. The molecular damage resulting from global stress can have detrimental effects on cellular function and viability, impairing various physiological pathways. Importantly, protein oxidation can cause protein damage, impair protein function, and promote protein aggregation, disrupting cellular homeostasis [4-7]. Therefore, it is critical for cells to employ efficient regulatory pathways to increase cellular viability under oxidative stress conditions.

To mitigate the harmful effects of excessive ROS on proteins, eukaryotic cells invoke a series of regulatory mechanisms through the reprogramming of gene expression at the transcription and translation levels. While many studies have identified and characterized ROS-responsive transcriptional reprogramming [8-12], the detailed mechanisms by which translation is regulated during oxidative stress remain elusive [13-17]. Translation, a key aspect of the central dogma of molecular biology, controls cellular dynamics and functioning by regulating protein synthesis [16]. Dysregulation of protein translation can impair healthy cellular physiology, constituting various diseases such as cardiovascular disease, neurodegenerative diseases, and cancers [1, 16]. Therefore, to maintain cellular homeostasis under different conditions, translation is tightly controlled through various mechanisms, such as post-translational modification of initiation factors, depending on cellular needs [14, 16-18].

Translational regulation during oxidative stress involves a complex myriad of targeted pathways, including controlling translation initiation, elongation, and termination [13]. Typically, cells induce a global inhibition of protein synthesis, potentially limiting gene expression under error-prone stress conditions while promoting the synthesis of essential antioxidant proteins [6, 7, 13]. However, most extant studies on stress-induced translational regulation focus on the mechanisms by which translation initiation is regulated, while little is known about regulation of translation elongation [6, 13]. For example, a large body of evidence shows that during oxidative stress, a key yeast protein kinase, Gcn2, is activated and phosphorylates the eukaryotic translation initiation factor elF2α, which impairs new cycles of translation initiation [6, 19]. Over time, developments in genome-wide studies that characterize protein dynamics during translation have shown evidence of potential post-initiation stages of stress-induced regulation, sparking rising interests in ROS-responsive translation elongation control [20]. Specifically, some studies suggest that excessive ROS may affect elongation speed, mediate the degradation of key tRNA molecules, and cause ribosomal pausing [4, 15, 21]. However, few studies have identified ROS-responsive translation elongation regulatory enzymes or characterized the detailed mechanisms by which this regulation occurs.

Our lab recently discovered a novel mechanism for regulating translation elongation during oxidative stress in *Saccharomyces cerevisiae* (budding yeast), a well-studied eukaryotic model that is useful for analyzing molecular mechanisms of the oxidative stress response [14, 22]. Previously, research has shown a global accumulation of polyubiquitin in yeast during the oxidative stress response [23, 24]. Ubiquitination, the covalent attachment of the small, highly conserved protein—ubiquitin—to target molecules, is an important post-translational modification that regulates protein dynamics by signaling various cellular pathways such as proteasomal degradation, subcellular localization, and membrane transport [18, 24, 25]. Due to its wide range of cellular signaling capabilities, specificity of ubiquitination depends on ubiquitin's ability to generate unique linkage types that influence different pathways [26]. Ubiquitin forms chains by attaching to different lysine residues on multiple ubiquitin molecules. These chains, denoted by the lysine residue each ubiquitin molecule is attached to (e.g. K63 is a linkage type in which individual ubiquitin molecules attach to the lysine at the 63rd position in the ubiquitin protein sequence), generate specific motifs that are recognized by proteins that carry out different functions [25, 26]. Much of the stress induced accumulation has traditionally been attributed to the well-studied role of K48-linked polyubiquitin in signaling the proteasomal degradation of proteins [23, 27, 28]. However, using mass spectrometry, we recently showed that K63-linked polyubiquitin chains (K63ub), a less-studied proteosome-independent modifier, heavily accumulates on ribosomes in response to oxidative stress [14]. However, the mechanism by which this accumulation occurred remained unknown.

Stress-induced translational control provides immediate and effective changes in protein levels that are required for cells to maximize survival during oxidative stress [18]. Ribosomes play an essential role in controlling protein levels by mediating all protein synthesis. Therefore, small changes in ribosomal activity can have significant effects on global protein synthesis [18]. During oxidative stress, we found that the ubiquitination of ribosomes arrests ribosomes at the pre-translocation stage of translation elongation, serving as a post-initiation modulator of translation during oxidative stress [14, 15]. This ubiquitin-mediated response, named the redox control of translation by ubiquitin (RTU) pathway, could be a protective measure, potentially stalling ribosomes selectively at error-prone mRNA sequences which may decrease the cellular burden of misfolded protein aggregation [3, 15]. Furthermore, stalling ribosomes may contribute to the global inhibition of protein production attributed to the general oxidative stress response. Because protein synthesis is energetically costly, global translation repression via ribosomal stalling may accelerate the stress response by allocating energy expenditure on the production of antioxidants [3, 14, 15]. Research shows that under peroxide treatment, ROS levels are higher in cells in the absence of stress-induced translation control, supporting the potential protective role of ubiquitin-mediated ribosomal stalling [3]. Because ubiquitin is a post-translational modulator of ribosome activity, it can have numerous effects on ribosomal functioning and abundance [18]. Understanding the regulation of ubiquitination under oxidative stress could elucidate potential regulatory roles of post-translational modification in promoting cellular health. However, the mechanism by which this stress response is mediated by ubiquitination has only begun to be elucidated.

Following the canonical pathway of protein ubiquitination, the ubiquitin-mediated modification of targeted proteins is catalyzed by a ubiquitin activating enzyme (E1), conjugating enzyme (E2), and ligase (E3) that adds ubiquitin to its substrate, and is reversed by a deubiquitinating enzyme (DUB) that cleaves ubiquitin from its substrate [26]. This cascade of enzymatic regulation controls the levels of protein ubiquitination within a cell (Figure 1A). Previously, we identified Rad6-Bre1 as the E2-E3 pair that modifies ribosomal proteins during oxidative stress [14]. Furthermore, upon screening for potential deubiquitinating enzymes (DUBs) implicated in the RTU pathway, we identified Ubp2 as the DUB responsible for regulating deubiquitination during stress by cleaving K63ub from ribosomal proteins during oxidative stress recovery [14]. We determined that the deubiquitinating activity of Ubp2 is redox inhibited by H_2O_2, leading to the observed accumulation of K63ub on ribosomes that stalls translation during oxidative stress (Figure 1B) [14]. Furthermore, we found that Ubp2 removes K63ub from ribosomes during stress recovery, leading to a rapid decline in K63 polyubiquitination shortly after the onset of oxidative stress (Figure 1C) [14]. Although we have identified how Ubp2 is implicated in the oxidative stress response, the mechanisms by which Ubp2's activity is regulated during stress recovery remain elusive.

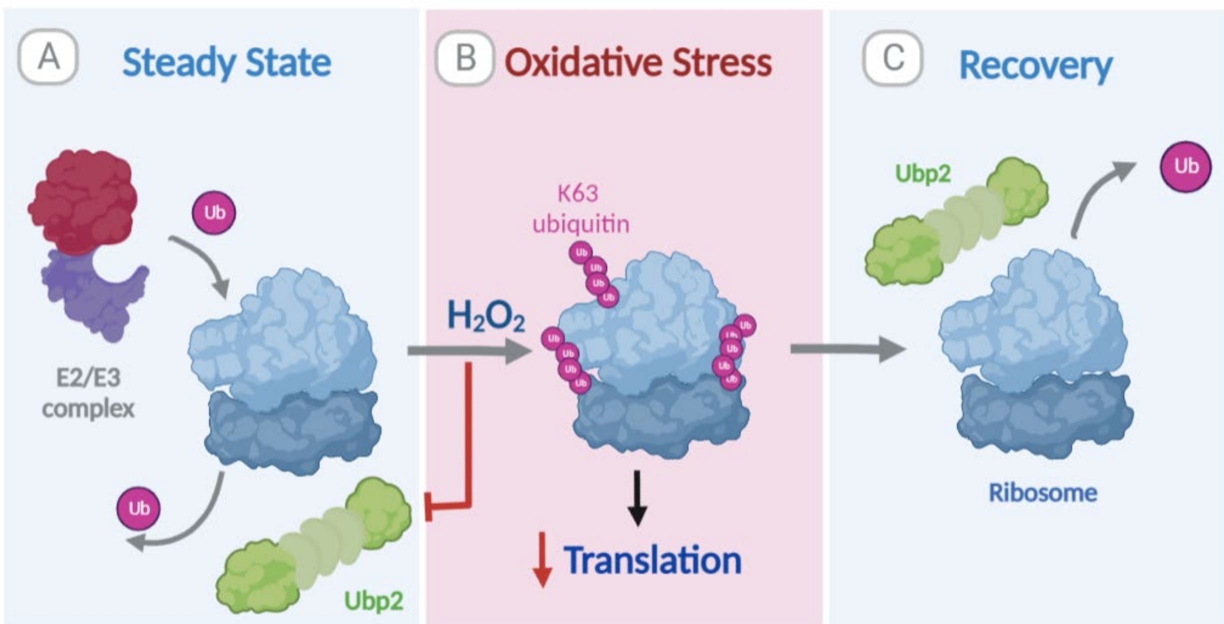

Figure 1. Redox Control of Translation by Ubiquitination (RTU) Pathway. This diagram depicts the proposed mechanism by which K63ub of ribosomes is regulated normally, during oxidative stress, and during stress recovery. (A) Normally, ubiquitination of ribosomes is regulated by an E2/E3 complex that adds K63ub onto ribosomes and Ubp2, a deubiquitinating enzyme (DUB) that removes K63ub from ribosomes. (B) Upon exposure to H_2O_2, Ubp2 is inhibited leading to an accumulation of K63ub on ribosomes that stalls translation. (C) However, during recovery, Ubp2 removes K63ub from ribosomes.

Ubp2, a cysteine protease DUB, is a member of the ubiquitin-specific protease (UBP) DUB family in yeast (USP in humans), which rely on reactive cysteine residues at their catalytic site for functioning and activity [18, 24, 26]. Due to their catalytic cysteine, it is proposed that some members of the UBP DUB family are potentially susceptible to redox-regulation [18].

Recently, research showed that only certain cysteine protease DUBs are susceptible to redox-regulation by hydrogen peroxide, an oxidizing agent, and dithiothreitol (DTT), a reducing agent [29]. However, the structural aspects that determine which cysteine proteases change activity in the presence of redox agents remain elusive. Recently, we found that Ubp2's activity is inhibited by hydrogen peroxide, and rescued by DTT *in vitro*, suggesting that it is capable of being redox-regulated [14]. However, the redox-regulation of Ubp2 activity *in vivo* remains to be characterized. Furthermore, little is known about how its structural elements govern its regulation during oxidative stress.

Ubp2 may be susceptible to redox regulation at its catalytic cysteine during oxidative stress *in vivo* because its catalytic cysteine can potentially undergo different oxidation states (i.e., reversible disulfide bonds and sulfenic acids, or irreversible sulfinic and sulfonic acids) [21] that potentially alter enzymatic function when exposed to oxidative conditions [3]. Researchers speculate that exposure of this catalytic cysteine to its substrate (K63ub) may be mediated by specific domains that help recognize its protein target, conformational arrangements of catalytic domains, and acidity of the active site [18, 21, 26]. However, the structure of Ubp2 has not been characterized in previous literature. Due to this, it is difficult to determine how oxidative conditions change Ubp2's structure and how these changes govern Ubp2's response during stress.

Therefore, the aim of this investigation is to elucidate how Ubp2 is regulated during the oxidative stress response, specifically investigating:

1. How is K63ub accumulation reversed during oxidative stress recovery?
2. How does Ubp2's structure (domains & cysteine residues) regulate its function during oxidative stress?

Because K63ub is removed from ribosomes shortly after the onset of stress, I hypothesized that the removal is a result of the reactivation of the existing pool of inactivated Ubp2 enzymes. Alternatively, I hypothesized that this removal is a result of the synthesis of new, active Ubp2 enzymes. Using cycloheximide assays and western blot, I showed that Ubp2 removes K63ub independently of translation, thus suggesting that its activity is reversed after stress cessation. To better understand how Ubp2 is reactivated during stress recovery, I analyzed structural models of Ubp2, identifying conserved domains that may be required for activity and regulation. Using western blot and cellular assays on mutant yeast cells with truncated *UBP2* genes, I characterized the regulatory functions of Ubp2's conserved structural domains generally and during the oxidative stress response. Furthermore, I characterized the stress-induced regulatory role of Ubp2's non-catalytic cysteine residues, which potentially offer an additional layer of stress-induced regulation due to their sensitivity to oxidation.

Therefore, this study suggests a novel mechanism by which Ubp2 regulates ribosomal K63-polyubiquitination in the translational oxidative stress response and establishes a basis for characterizing structural elements that govern the stress-induced activity and regulation of DUBs. Understanding the mechanism by which Ubp2 is implicated in the oxidative stress response could elucidate a more comprehensive understanding of cellular resistance to oxidative stress, informing potential regulatory pathways of DUBs during oxidative stress.

METHODS

Strain generation and cultures

All *Saccharomyces cerevisiae* strains used in this study were grown at 200 rpm agitation at 30°C in synthetic dextrose minimal media with dropout amino acid supplements (SD: 67% yeast nitrogen base, 2% dextrose, and required amino acids) without uracil (SD-ura) for selective growth. To generate mutant strains, a yeast strain deleted for *UBP2* was transformed with the pYES2 plasmid where the Ubp2 full length sequence together with its promoter sequence were cloned to complement the genomic deletion. For this investigation, point mutations (C745S, C645S, and C621S) and truncations with different combinations of repeated domain 1 (RD1), repeated domain 2 (RD2), and repeated domain 3 (RD3) were created in the Ubp2 sequence. All mutated/truncated plasmids were then transformed in the same yeast strain deleted for *UBP2*. The following strains were generated by Vanessa Simões: Ubp2-HA, denoted Ubp2 full length (Ubp2 FL) for the purpose of this investigation and Ubp2 (C745S). The following strains were generated by Blanche Cizubu: Ubp2 RD1-3, Ubp2 RD2-3, Ubp2 RD3, Ubp2 RD2, Ubp2 RD1-2, Ubp2 RD2-3 (C745S), Ubp2 RD2-3 (C645S), and Ubp2 RD2-3 (C621S).

Cell cultures were pre-inoculated in 5 mL of SD-ura media at 30°C overnight and then diluted to an optical density measured at the wavelength 600 nm (OD600) of 0.001. All cells were treated at log phase (OD600 ~ 0.3–0.5). Unless noted otherwise, the cultures were treated with freshly diluted hydrogen peroxide (Sigma, #216763), achieving a final concentration of 0.6 mM.

Experimental Models

Cycloheximide Chase Assay

Cells were treated with 150 mM cycloheximide (Sigma-Aldrich) independently or in addition to hydrogen peroxide and grown at 200 rpm agitation at 30°C for 90 minutes. Cells were collected via centrifugation at 4°C in 30-minute intervals. Experiment performed by Nate Snyder.

K63ub Time course experiments

Cells were treated with hydrogen peroxide and grown at 200 rpm agitation at 30°C for 30 minutes. Cells were then washed with 1 mL deionized (DI) water and resuspended in 2 mL of fresh SD-ura media before being added to 75 mL of fresh media and grown at 200 rpm agitation at 30°C. Cells were collected via centrifugation at 4 °C.

Western blotting

Cells were lysed at 4 °C in standard buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM iodoacetamide (IAM), 2× EMD Millipore protease inhibitor cocktail set I, and DI water. The protein concentration was determined by Bradford assay (Bio-Rad). Unless noted otherwise, all proteins were diluted to a final concentration of 1.0 μg/μL. Proteins were then separated by standard 10% SDS-PAGE loaded in Laemmli buffer at 150 volts for 60 minutes and transferred to PVDF membrane (ThermoFisher). Membranes were blocked in 5% milk in Tris Buffered Saline Buffer with Tween 20 (TBST) or 5% bovine serum albumin in TBST. The antibodies used for this work were antibodies for K63 ubiquitin (1:8000, EMD Millipore, cat. no. 05-1308, clone apu3), GAPDH (1:3000, Abcam, cat. no. ab9485), HA (1:3000, ThermoFisher, cat. no. 71-5500), and Rabbit IgG (1:4,000-10,000; Cytiva, cat. no. NA934) which were conjugated with HRP and ELC detection reagents (Cytiva). All antibodies were validated by the manufacturers.

Growth assays

Cell cultures were grown in SD-ura to log phase and back-diluted to OD600 0.1. For untreated cells, equal volumes of cell suspension and fresh SD-ura media (75 μL) were added to a 96-well plate in triplicate. For treated cells, 75 μL of cell suspension, 200 μg/mL of ADCB, and the remaining volume of fresh SD-ura media (to make 150 μL solutions) were added to a 96-well plate in triplicate. Plates were incubated at 30°C for 4 days with the absorbance (OD600) measured every 15 minutes in a Tecan Sunrise microplate reader.

3D structural analysis

All 3D graphical images of the yeast Ubp2 structure were generated from the Alphafold protein database (UniProt ID: A0A6C1E829) and I-TASSER using the PyMOL molecular visualization software.

RESULTS

K63-linked polyubiquitin chains are removed independently of translation during stress recovery

To better understand how K63ub accumulation is reversed during oxidative stress recovery, we tested whether *de novo* translation of Ubp2 is required to remove K63ub during stress recovery. We treated wildtype (WT) yeast cells with cycloheximide to block translation and hydrogen peroxide to induce stress, and measured stress recovery over time. Cells were collected in intervals of 30 minutes for 90 minutes. The results showed that K63ub is removed independently of translation, suggesting that newly synthesized Ubp2 proteins are not required to remove peroxide-induced K63ub accumulation (Figure 2). Therefore, these results suggest that K63ub is removed due to the reactivation of the existing pool of inactivated Ubp2 enzymes. However, the mechanism by which this reactivation occurs remained unknown.

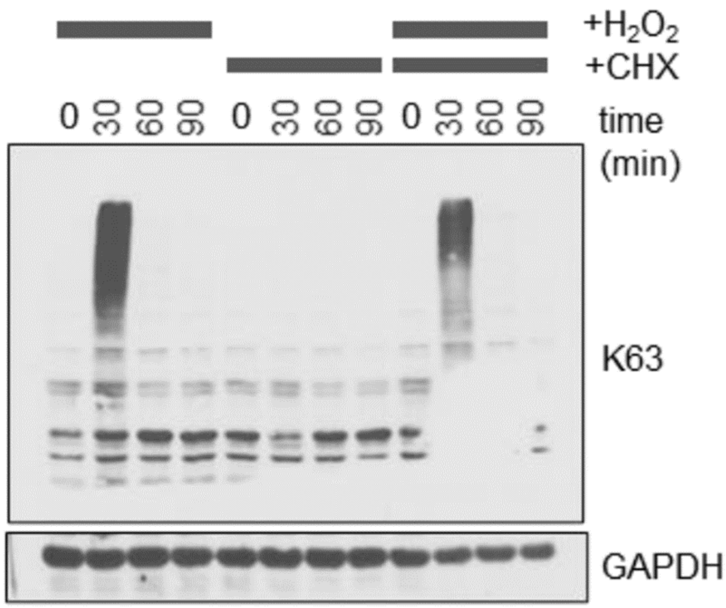

Figure 2. K63ub is removed independently from translation. WT yeast cells are treated with 0.6 mM H_2O_2, 150 mM cycloheximide, or both reagents for 90 minutes. Samples were lysed and processed on western blot using K63, HA and GAPDH antibodies.

Ubp2 has three short, evolutionarily conserved repeated domains next to its catalytic domain

To better understand how Ubp2 potentially regains activity during stress recovery, I identified key structural domains and cysteine residues that may be important for Ubp2's function. Protein domains are structurally and functionally independent units that contribute to a protein's range of function [32], so it was possible that they are important for Ubp2's activity and regulation. Additionally, cysteine residues can take on different oxidation states that regulate their functionality, making them especially susceptible to redox regulation [21], so I asked if there are important cysteines in Ubp2's structural domains. Using the InterPro protein database to analyze Ubp2's potential structural domains [30], I found that Ubp2 has three, short evolutionarily conserved repeated domains--repeated domain 1 (RD1), repeated domain 2 (RD2), and repeated domain 3 (RD3)—which hold two cysteine residues (C621 and C645) (Figure 3A). These domains are directly adjacent to Ubp2's catalytic domain at its C-terminus, which holds its catalytic cysteine (C745) and two other cysteine residues (C821 and C944) (Figure 3A). The N-terminus does not seem to have distinct conserved domains (Figure 3A). These results suggest that Ubp2 has at least four unique domains, all of which could be important for its activity.

I then asked how these predicted domains map out spatially onto a 3D model of Ubp2's structure to better inform hypotheses regarding potential domain interactions and dynamics. However, the structure of Ubp2 has not yet been characterized. Therefore, using AlphaFold's predicted structure of Ubp2 [31], and the PyMOL molecular visualization software, I aligned the InterPro's proposed domains with the predicted structure and generated a domain-based structural model of Ubp2 (Figure 3B). Repeated domains are a type of evolutionarily conserved protein combination of domains from the same family in tandem [32]. Research suggests that they are often involved in interactions with proteins or other ligands and provide proteins with variability in activity and function [32]. Given this and their proximity to Ubp2's catalytic domain (and cysteine), I asked whether RD1, RD2, and RD3 have specific regulatory functions on Ubp2's activity during stress recovery. To test this, we generated *UBP2* truncated mutants with different combinations of these domains, which will be used in subsequent experiments to investigate the regulatory role of each domain on Ubp2's activity (Figure 2C).

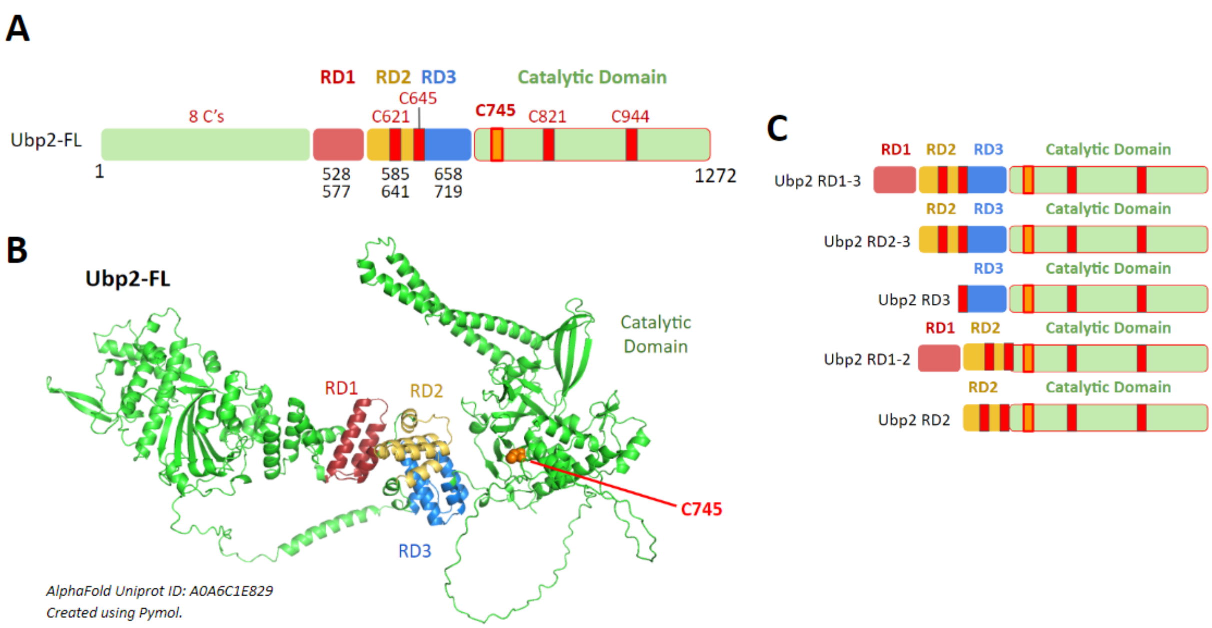

Figure 3. Ubp2 has three short, evolutionarily conserved repeated domains next to its catalytic domain. (A) Schematic of Ubp2-FL protein construct generated using InterPro's proposed domains. (B) Predicted structure of Ubp2-FL with unique domains, generated using Alphafold protein database and PyMOL molecular visualization software. (C) Schematics of Ubp2 truncated mutants' constructs.

Repeated domains regulate Ubp2's activity in the cellular uptake of ADCB

To characterize the regulatory roles of Ubp2's repeated domains on its function, I investigated their effects on a pathway that is known to require Ubp2's function. L-azetidine-2-carboxylic acid (ADCB) is a toxic proline analogue that causes cellular damage and inhibits cellular growth [33]. Although the exact mechanism has not fully been elucidated, studies suggest that Ubp2 inhibits the cellular uptake of ADCB by deubiquitinating its transporter [34] . When Ubp2 is inactive, such as with Ubp2 (C745S), the transporter is active, and cells are more sensitive to ADCB resulting in cellular growth deficits (Figure 4A, right). However, when Ubp2 is fully active, such as with Ubp2-FL, the transporter is inactivated, and cells are less sensitive to ADCB resulting in cellular growth (Figure 4A, left). Therefore, I performed cellular growth assays on the truncated Ubp2 mutants, using cellular growth under ADCB treatment, measured in absorbance over time, as a proxy measurement for Ubp2 activity.

First, I investigated the independent effect of the truncated *UBP2* mutations on cellular growth. There were no significant differences in the growth rates between strains, suggesting that the mutations alone do not affect cellular growth (Figure 4B). Next, I investigated the effect of each repeated domain on Ubp2's activity during ADCB stress. Strains with full Ubp2 enzymes showed the highest growth rates while a mutation in the catalytic cysteine resulted in significant growth deficits (Figure 4C). While removal of the N-terminus resulted in intermediate growth rates, subsequent removal of RD1 rescued growth deficits to levels comparable to the strain with full Ubp2 enzymes (Figure 4C). This suggests that RD1 inhibits Ubp2's ability to deubiquitinate ADCB transporters. However, subsequent removal of RD2 resulted in growth deficits, suggesting that RD2 enhances Ubp2's ability to deubiquitinate ADCB transporters (Figure 4C). The effects of RD3 on Ubp2's activity remained unclear.

To better understand how RD3 regulates Ubp2's activity, I asked whether RD2 increases Ubp2's activity independently or whether RD2 and RD3 are both required to rescue full activity. I found that strains with only RD2 showed growth deficits compared to strains with both RD2 and RD3, suggesting that RD3 is required in conjunction with RD2 to enhance Ubp2's activity (Figure 4D). These results suggest that RD3 may serve as a structural linker between RD2 and the catalytic domain, meditating the interaction between the two domains. Furthermore, RD1 increased sensitivity to ADCB, supporting that it serves as an inhibitory domain in the ADCB pathway (Figure 4D). However, it remained unclear how these regulatory domains affect Ubp2's activity during oxidative stress recovery.

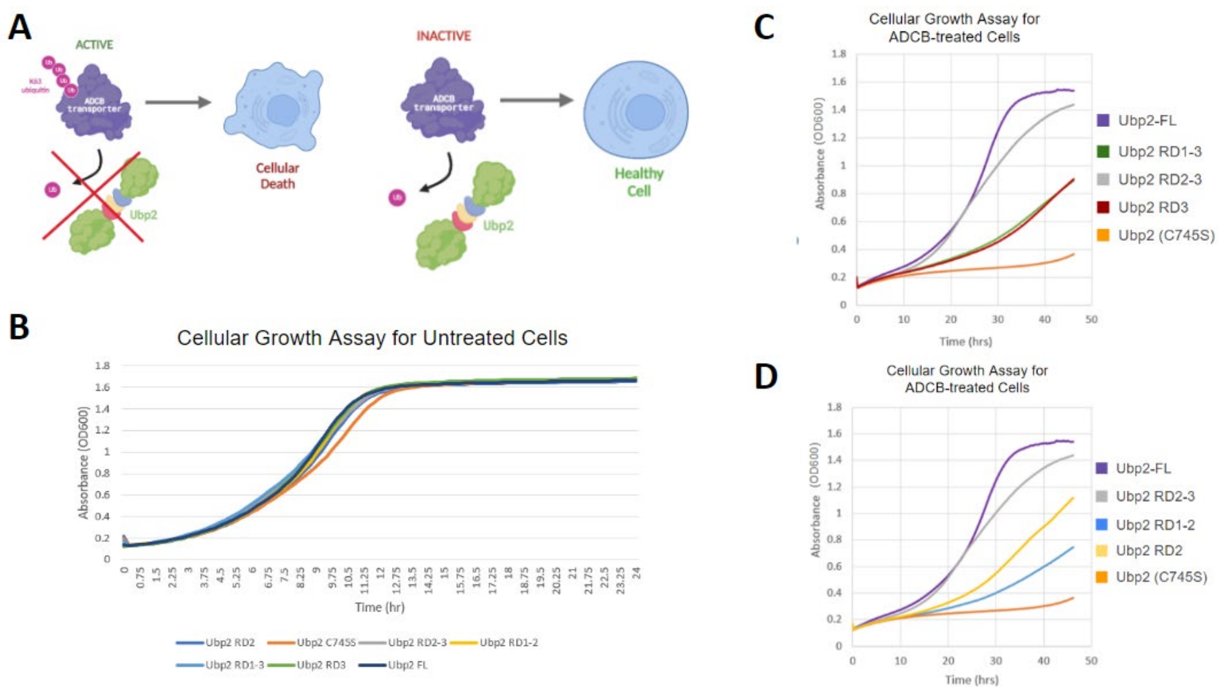

Figure 4. Repeated domains regulate Ubp2's activity in the cellular uptake of ADCB. (A) Schematic of Ubp2's role in cellular uptake of ADCB. (B) Cellular growth assay for untreated Ubp2 truncated mutants. (C-D) Cellular growth assay for treated Ubp2 truncated mutants. Cellular growth measured in Absorbance (OD600).

Repeated Domain 1 inhibits Ubp2's ability to remove K63ub during oxidative stress recovery

Because the repeated domains regulated Ubp2's ability to deubiquitinate the ADCB transporter, I hypothesized that RD1 would inhibit Ubp2's ability to remove K63ub during stress recovery and that RD2-RD3 would promote Ubp2's ability to remove K63ub during oxidative stress recovery. To test if the repeated domains regulate Ubp2's activity during oxidative stress, I treated Ubp2 RD2-3 and Ubp2 RD1-3 strains with hydrogen peroxide and measured stress recovery over time. I showed that RD2 linked to RD3 promotes Ubp2's ability to quickly remove K63ub during stress recovery (Figure 5A). However, RD1 significantly inhibits Ubp2's ability to remove K63ub accumulation during stress recovery (Figure 5B). These results suggest that RD2 linked to RD3 play an activating role and RD1 plays an inhibitory role in the redox regulation of Ubp2's activity.

Furthermore, I found that the Ubp2 RD2-3 strain forms a disulfide bond (denoted by a double band under HA-detection) upon stress induction, which corresponds to K63ub accumulation levels (Figure 5A). The double bands potentially indicate that two forms of Ubp2 RD2-3 were detected because of the proximity of the bands. It is likely that the disulfide bond changes the conformation of Ubp2 which affects how it travels through the SDS-Page gel, resulting in a proximal band near the expected Ubp2 band. However, RD1 seems to inhibit the formation of this disulfide, suggesting that disulfide bond formation may contribute to Ubp2's ability to remove K63ub during stress recovery. Because cysteines are susceptible to redox-regulation, it is possible that the formation of a disulfide bond offers an additional level of regulation for Ubp2's activity during stress recovery. However, it remained unclear whether RD1 inhibits Ubp2's ability to form a disulfide bond more generally.

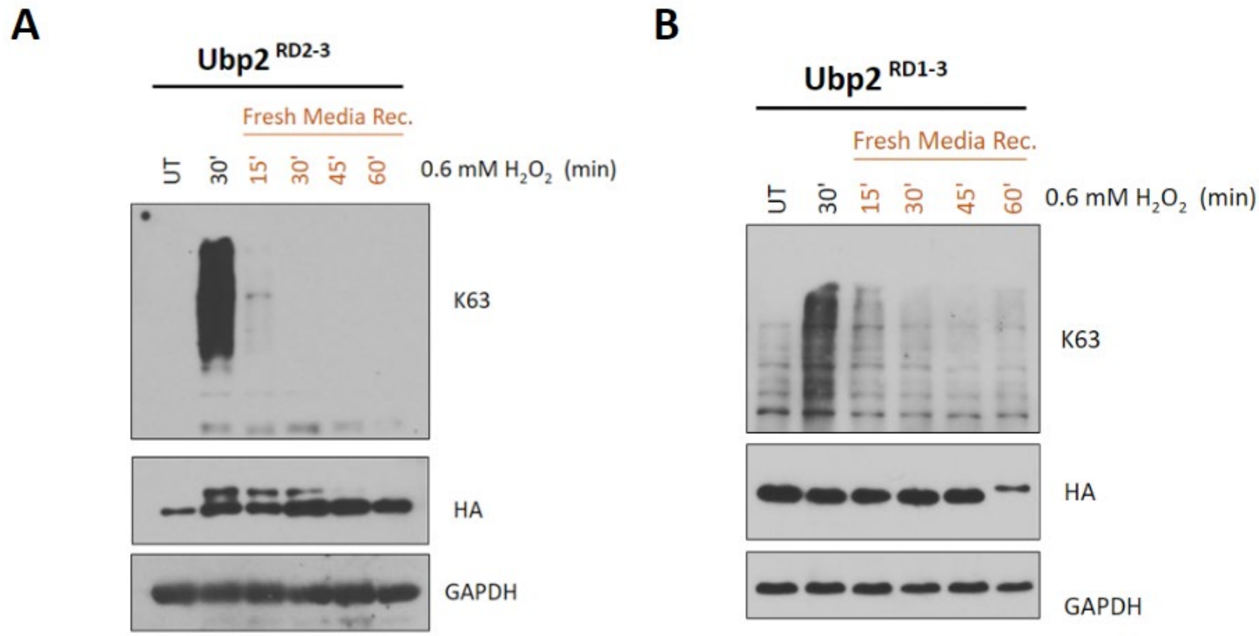

Figure 5. Repeated Domain 1 inhibits Ubp2's ability to remove K63ub during oxidative stress recovery. (A) Ubp2 RD2-3 stress recovery. (B) Ubp2 RD1-3 stress recovery. Samples treated with 0.6 mM H2O2 for 30 minutes. Samples inoculated in fresh media at collected after 15 min, 30 min, 45 min, and 60 min. Samples processed on western blot using K63, HA, and GAPDH antibodies.

Repeated Domain 1 decreases the formation of peroxide-induced disulfide bond

To test whether RD1 inhibits formation of the disulfide bond during stress, I treated different *UBP2* mutant strains with hydrogen peroxide and tested for the presence of the disulfide bond. The results show that RD1 greatly reduces the disulfide bond formation during oxidative stress (Figure 6). Additionally, a mutation of Ubp2’s catalytic cysteine (C745) inhibits the formation of the disulfide bond, suggesting that C745 may be one of the cysteines involved in the bond (Figure 6). Overall, these results suggest that RD1 inhibits the formation of a disulfide bond between Ubp2’s catalytic cysteine and another cysteine residue.

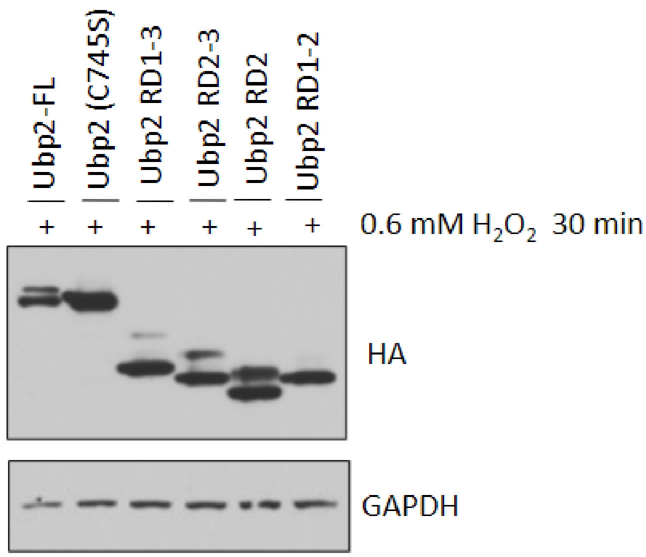

Figure 6. RD1 decreases the formation of peroxide-induced disulfide bond. Samples treated with 0.6 mM H_2O_2 for 30 minutes. Samples were lysed and processed on western blot using HA and GAPDH antibodies.

Ubp2's catalytic cysteine forms a disulfide bond during oxidative stress

To better understand the potential role of the disulfide bond in Ubp2's activity during stress, I then asked which cysteine residues are involved in the bond. Because strains with only RD2 and the catalytic domain formed the disulfide bond, I hypothesized that the one of cysteine residues within RD2 (C621 and C645) participate in the bond during stress. Furthermore, it is likely that one or multiple of these residues form a disulfide bond with the catalytic cysteine (C745) since a mutation of the catalytic cysteine resulted in the inhibition of the disulfide bond formation (Figure 6). Therefore, I treated different cysteine residue Ubp2 RD2-3 mutant strains with hydrogen peroxide and tested for the presence of the disulfide bond. Similarly to the previous experiment, a mutation in the catalytic cysteine inhibited the formation of a disulfide bond, supporting that C745 is one of the residues that involved in the bond (Figure 7). However, C645 and C621 are not directly involved in the formation of the disulfide bond (Figure 7). The results suggest that C645 may indirectly inhibit some level of disulfide bond formation because a mutation in this cysteine resulted in more disulfide bond formation (Figure 7). It is possible that C645 promotes a structural conformation that limits the exposure of the catalytic cysteine to other cysteine residues. However, the C645S bond formation was not reproducible in other experiments, so it likely does not indirectly affect disulfide bond formation. Overall, these results suggest that the catalytic cysteine forms a disulfide bond with another cysteine within Ubp2's catalytic domain (C944 or C821). Furthermore, the addition of DTT, a reducing agent, reversed the formation of the double band, supporting that this represents disulfide bond formation (Figure 7).

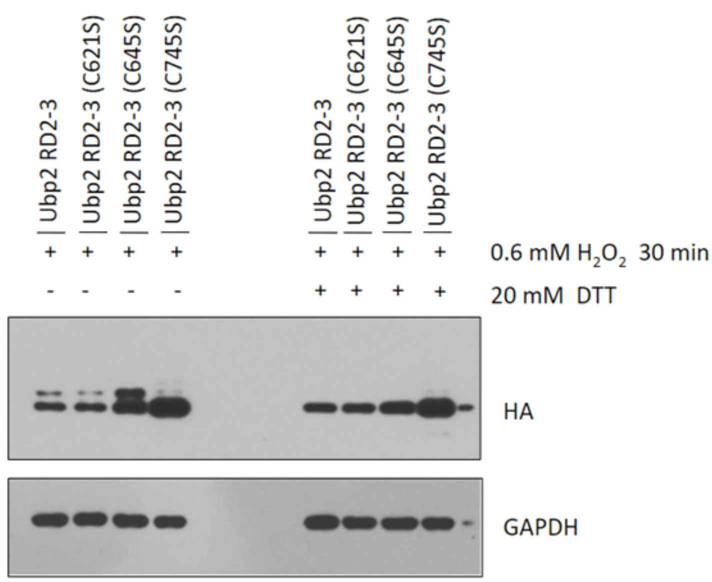

Figure 7. Ubp2's catalytic cysteine forms a disulfide bond during oxidative stress. Samples treated with 0.6 mM H_2O_2 for 30 minutes in the presence or absence of 20mM DTT. Samples were lysed and processed on western blot using HA and GAPDH antibodies.

RD1 changes the conformation of the catalytic site in predicted models

To better understand the mechanisms by which the repeated domains affect Ubp2's activity and which cysteine residues contribute to the peroxide-induced disulfide bond, I used the I-TASSER structure prediction software to generate the predicted structures of the sequences for

Ubp2 FL, Ubp2 RD2-3, and Ubp2 RD1-3 [35] (Figure 8). I then asked if the repeated domains affect the structure of the catalytic site, specifically with regards to the positioning of the catalytic cysteine and other cysteine residues in the catalytic domain. Because the average disulfide bond length is 2.0-3.0 Å [36], I hypothesized that RD1 increases the distance between the catalytic cysteine and C944 and C821, contributing to the inhibition of the disulfide bond during stress. The models show that RD1 does not significantly change the C745-C821 bond distance compared to full Ubp2 enzymes, nor the C745-C944 bond distance compared to Ubp2 RD2-3 enzymes (Figure 8). These results suggest that RD1 may not be inhibiting the disulfide bond formation by increasing the distance between the cysteine residues. There might be another mechanism by which this inhibition occurs that is not captured in these models. However, the model suggests that RD1 changes the conformation of the catalytic site, making the catalytic cysteine more exposed (Figure 8B). It is possible that RD1 changes the interaction between Ubp2 and its substrate (K63ub) at the active site, contributing to its inhibitory effect. However, future experiments are required to investigate the mechanisms by which this occurs and to elucidate a better understanding of the interprotein interactions that may be occurring.

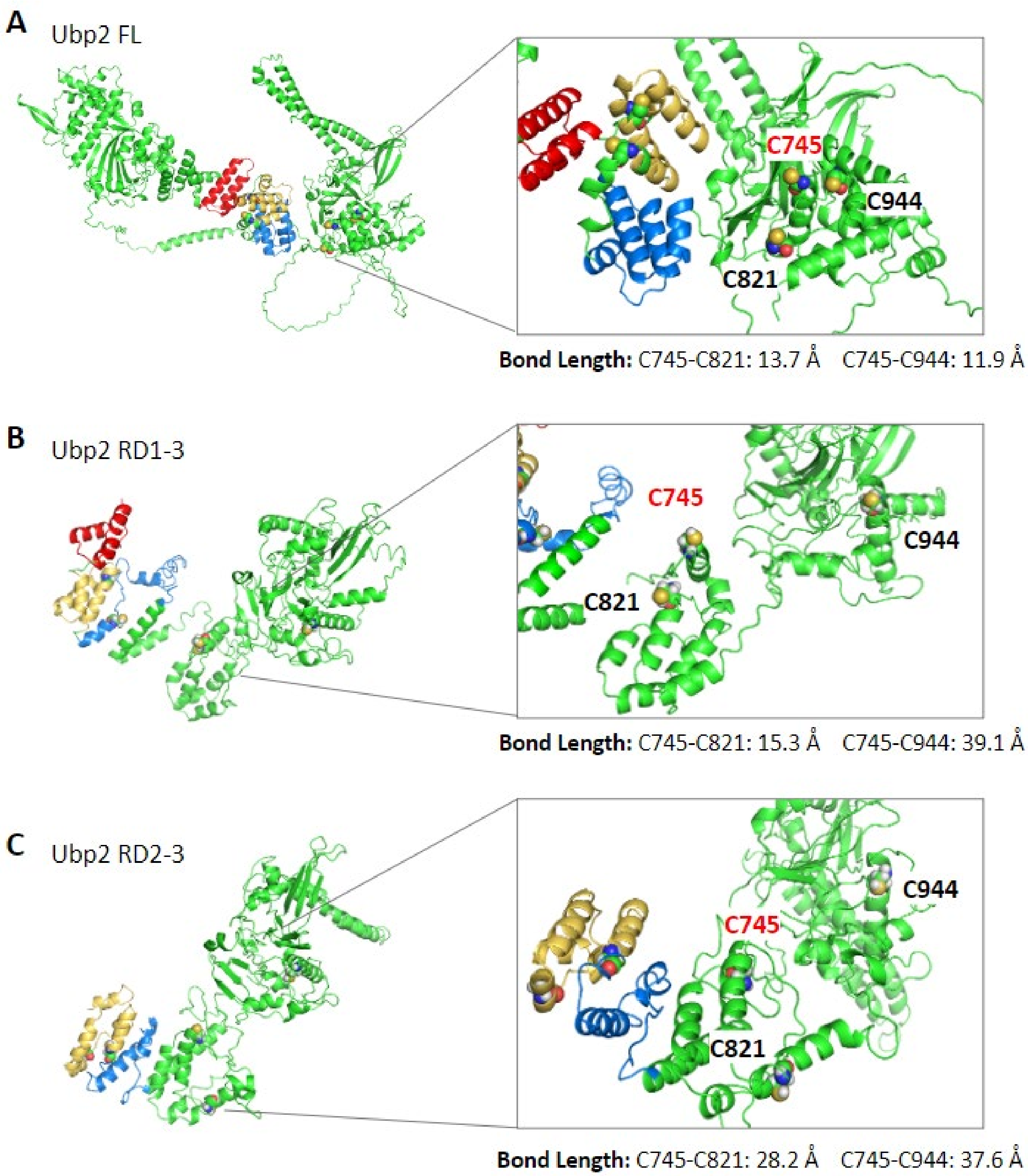

Figure 8. RD1 changes the conformation of the catalytic site in predicted models. Structural detail of (A) Ubp2 FL, (B) Ubp2 RD1-3, and (C) Ubp2 RD2-3 at the catalytic domain. Predicted positions of C745, C821, and C944, generated using I-TASSER structure prediction software and aligned using PyMOL molecular visualization software.

DISCUSSION

Previously, Ubp2 has been proposed to act as an oxidative stress sensor in the RTU pathway because its inhibition during stress results in an accumulation of K63ub on ribosomes, stalling translation elongation [37]. *In vitro* studies showed that the hydrogen peroxide inhibition of Ubp2 is reversed with the addition of a reducing agent, DTT, suggesting that Ubp2 may be susceptible to redox regulation *in vivo* [14]. Given this, it was likely that Ubp2 falls into a distinct class of cysteine protease DUBs whose activity is redox-regulated. However, the structural aspects that determine which DUBs constitute this redox-regulated group had yet to be characterized. Furthermore, the mechanism by which Ubp2 removes K63ub during stress recovery remained elusive. In this study, I characterized novel roles for important structural domains and cysteine residues that regulate Ubp2's activity during oxidative stress recovery, which contribute to its ability to regulate K63ub dynamics in the acute oxidative stress response.

First, I determined that the removal of K63ub accumulation during stress recovery does not require translation of new Ubp2 proteins. Because Ubp2 is inhibited by hydrogen peroxide, it was possible that this inhibition was due to the degradation of damaged Ubp2 enzymes. By determining that K63ub can be removed without the synthesis of new Ubp2 molecules, I have expanded the possible mechanisms that regulate the stress response. It is likely that the reversibility of Ubp2's inhibition is due to an acute inhibition of its activity, rather than complete degradation and de novo synthesis. This suggests that Ubp2 is reactivated through redox regulation. Understanding how this reactivation occurs can inform the mechanisms by which other DUBs and enzymes are regulated during stress recovery. To characterize potential mechanisms by which this occurs, I identified four unique domains within Ubp2's structure that affect its activity, including three conserved repeated domains and a catalytic domain which holds Ubp2's catalytic cysteine. Repeated domains are often involved in protein binding and signaling in order to mediate protein-protein interactions and fundamental functioning [38]. First, I identified that RD1 is an inhibitory domain whose regulation of Ubp2's activity likely results in a less efficient response to peroxide stress and longer ribosomal stalling periods, significantly limiting the cellular capacity to combat ROS. Additionally, RD2 is an activating domain, especially in conjunction with RD3, which may serve as an important linker domain, suggesting that the position of RD2 in relation to the catalytic domain also affects Ubp2's activity and the cellular capacity to combat ROS.

Given their proximity to the catalytic cysteine and the established role of repeated domains in protein interactions, it is likely that the repeated domains undergo peroxide-induced conformational changes or steric hindrances that change the catalytic cysteine's exposure to oxidation. Catalytic cysteines (pKa~8.5) rely on their deprotonated form (thiolate) for activity, which is mediated by the pH of the catalytic microenvironment [39]. Low pKa cysteine residues (pKa~4-6.5) tend to be more susceptible to oxidation [39]. Therefore, it is possible that RD1 lowers the pKa of Ubp2's catalytic cysteine by positioning positively charged amino acids, such as arginine and lysine, proximal to the catalytic cysteine during oxidative stress, making it more susceptible to irreversible oxidation and loss of function. Additionally, it is possible that the repeated domains influence K63ub binding directly. Preliminary data suggests that RD2, RD3, and the catalytic domain may hold three of K63ub binding domains. This potentially explains why having RD2 and RD3 is sufficient for the full functioning of Ubp2 during ADCB treatment and oxidative stress. It is possible that RD1 induces a conformational change that makes these binding sites less accessible and specific, ultimately inhibiting Ubp2's activity. Future experiments are required to explore these connections.

I then identified a novel linkage between Ubp2 disulfide bond formation and K63ub accumulation during oxidative stress. In active Ubp2 molecules, disulfide bonds form upon peroxide induction and progressively decline as K63ub accumulation declines. RD1 inhibits the initial formation of these disulfide bonds and shows persistent K63ub accumulation upon stress. Furthermore, I found that Ubp2's catalytic cysteine potentially makes this disulfide bond with another cysteine residue within the catalytic domain (C821 or C944). Collectively, these results suggest that the catalytic cysteine forms a protective disulfide bond with another cysteine residue in order to prevent further oxidation to irreversible forms of the inactive thiol group. It is likely that exposure of the catalytic cysteine to either of these cysteines is potentially regulated by conformational changes mediated by the repeated domains. Because RD1 is associated with the inhibition of Ubp2's activity, it is possible that its inability to promote the formation of a disulfide bond potentially contributes to this inhibition. Furthermore, the formation of the disulfide bond may contribute to Ubp2's ability to be reactivated during stress recovery. This is supported by the translation-independent removal of K63ub. If Ubp2's catalytic cysteine forms irreversible sulfinic and sulfonic acids, the catalytic cysteine would be permanently inactive and K63ub removal would require the synthesis of new Ubp2 enzymes. Because this is not seen in active Ubp2 strains, it is more likely that the disulfide bond protects from further oxidation, allowing for the reactivation of Ubp2 during stress recovery.

These results suggest that the formation of a reversible disulfide bond may be an integral part of the enzymatic regulation that governs the RTU pathway. Previously, we showed that the E1 and E2 responsible for adding K63ub onto ribosomes during stress, Rad6 and Uba1 respectively, forms a reversible disulfide bond at their catalytic sites that suppresses further ubiquitination of ribosomes during stress recovery [37]. Given that Ubp2 shows a similar formation of a reversible disulfide, it is possible that the redox regulation of the key regulators in this pathway employ similar mechanisms in which oxidative stress acts as in a switch-like manner to control the activity of these enzymes. This could be used to inform future studies on the regulation of the integrated RTU stress response. Furthermore, research suggests that many DUBs and E3 ligases interact and work in concert to regulate substrate ubiquitination as well as their own activity [40]. Because the cysteine residue that binds to Ubp2's catalytic cysteine to form the disulfide remained elusive, it is possible that Ubp2 forms a protective disulfide with Bre1, the E3 ligase implicated in the RTU pathway [14, 37]. This could inhibit both enzymes' activity upon stress induction and reverse their activity during recovery, which could inform the dynamics of this specific pathway.

Although this is the first concerted structural analysis of Ubp2's activity under oxidative stress conditions, there are many limitations to this study. The primary limitation is that Ubp2's structure has not been determined. Therefore, this study relied on domain predictions and molecular models to characterize Ubp2's domains and contextualize the patterns of activity present in the results. Predicted models can provide insight on structures that can inform hypotheses, but they carry a level of inaccuracy and uncertainty that limit the reliability of their use. There are many aspects that potentially regulate Ubp2's activity that cannot be captured by molecular models. For example, proteins are dynamic and go through many motions. However, molecular models only offer a snapshot of a protein in one position and form. Additionally, molecular models do not capture cellular protein-protein interactions that could be mediating regulation. Therefore, in the future, it will be necessary to employ cryo-EM microscopy to determine the structure of Ubp2 generally and during stress conditions. This would confirm or better contextualize the results of this study.

Despite the limitations, this study provides the framework to explore the redox-regulation of cysteine-based enzymes. Importantly, it raises many questions that can inform future projects. From the results, I hypothesized that Ubp2 forms a disulfide between the catalytic cysteine and another cysteine residue in the catalytic domain. To test if the peroxide-induced disulfide is an internal bond between Ubp2's cysteine, I would generate HA-tagged Ubp2 RD2-3 strains with cysteine residue mutations at position 821 and 944, treat them with peroxide, and determine if the disulfide forms with these mutations. If the disulfide is internal, I expect that one of these mutants would inhibit the formation of the disulfide bond. Preliminary data shows that Ubp2 (C944S) is unable to form a disulfide bond during stress, suggesting that this may be the other cysteine mediating the bond. However, it will be beneficial to perform this experiment using Ubp2 RD2-3 because it only contains five cysteine residues (two of which have been shown not to form the disulfide), whereas the Ubp2 FL contains the cysteine rich N-terminus which holds eight residues. Alternatively, I hypothesized that Ubp2 forms a disulfide with a potential cofactor, specifically Bre1. To test if it is an interprotein bond with Bre1, I would delete *BRE1* from the Ubp2-FL strain and determine whether the disulfide bond forms during stress. This could inform potential bond formation and protein-protein interactions between the two enzymes.

Overall, I have developed a framework for understanding the structural aspects that govern redox-regulated enzymes, specifically DUBs, during oxidative stress. Because oxidative stress causes global cellular damage, it has many negative physiological implications for stress-related diseases in humans [41]. Cells are highly susceptible to oxidative stress, which causes necrosis and apoptotic cell death that are characteristic of these diseases [41]. Therefore, understanding the ways by which ubiquitin-regulated pathways govern the cellular response to oxidative stress can inform potential therapies for stress-related diseases. Understanding how key enzymes, such as DUBs, are regulated during the stress response can inform potential therapies that modify their level of activity in order to increase cellular resistance to oxidative stress.

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