Structural Basis for Interaction between the Ubp3 Deubiquitinating Enzyme and Its Bre5 Cofactor*

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The Bre5 protein is a cofactor for the deubiquitinating enzyme Ubp3, and it contains a nuclear transfer factor 2 (NTF2)-like protein recognition module that is essential for Ubp3 activity. In this study, we report the x-ray crystal structure of the Bre5 NTF2-like domain and show that it forms a homodimeric structure that is similar to other NTF2-like domains, except for the presence of an intermolecular disulfide bond in the crystals. Sedimentation equilibrium studies reveal that under non-reducing conditions, the Bre5 NTF2-like domain is exclusively dimeric, whereas a disulfide bond-deficient mutant undergoes a monomer-dimer equilibrium with a dissociation constant in the mid-nanomolar range, suggesting that dimer formation and possibly also disulfide bond formation may modulate Bre5 function in vivo. Using deletion analysis, we also identify a novel N-terminal domain of Bre5 that is necessary and sufficient for interaction with Bre5 and use isothermal titration calorimetry to show that Bre5 and Ubp3 form a 2:1 complex, in contrast to other reported NTF2-like domain/protein interactions that form 1:1 complexes. Finally, we employ structure-based mutagenesis to map the Ubp3 binding surface of Bre5 to a region near the Bre5 dimer interface and show that this binding surface of Bre5 is important for Ubp3 function in vivo. Together, these studies provide novel insights into protein recognition by NTF2-like domains and provide a molecular scaffold for understanding how Ubp3 function is regulated by Bre5 cofactor binding.

The activity of enzymes that catalyze ubiquitin (Ub) transfer on target proteins is correlated with the regulation of many cellular processes including protein degradation, cell cycle control, stress response, DNA repair, immune response, signal transduction, gene regulation, endocytosis, and vesicle trafficking (1–3). Studies over the last decade have provided important mechanistic insights into catalysis and substrate specificity of Ub conjugation by the E1/E2/E3 multiprotein system (1). However, relatively little mechanistic information is available on the proteins that mediate deubiquitination, despite mounting evidence that these proteins play an important regulatory role in cellular processes as their Ub-conjugating counterparts (4).

Deubiquitination is catalyzed by deubiquitinating proteases, which are classified into five groups based on sequence homology: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific processing proteases (USPs in humans and UBPs in yeasts), OTU-domain ubiquitinaldehyde-binding proteins, Jab1/Pad1/MPN domain-containing metalloenzymes, and the ataxin-3-like proteases (5–11). In addition, sequence analysis yields an additional putative deubiquitylating protease family (12). Among the deubiquitinating protease families, UBPs represent the most widespread deubiquitylating enzymes across evolution. In particular, the Saccharomyces cerevisiae genome encodes for 16 UBPs and only one ubiquitin C-terminal hydrolase (13). In the human genome, there appears to be at least 4 and 63 distinctive genes encoding ubiquitin C-terminal hydrolases and UBPs/USPs, respectively (14).

The observation that UBPs are abundant and broadly conserved in many species including bacteria, yeast, and humans, suggests that they might play important specific roles in regulating diverse biological processes. The molecular basis for how different UBPs select their cognate substrates and mediate distinct cellular functions is still unclear. Unlike the highly conserved ubiquitin C-terminal hydrolases, the UBP family exhibits homology only in two regions that surround the catalytic Cys and His residues, thus called the Cys box (60–90-amino acid) and His box (60–90-amino acid) regions. Homology among the UBP enzymes is restricted to a roughly 350-residue catalytic core domain, whereas the UBPs contain variable N-terminal extensions, occasional C-terminal extensions, or insertions in the catalytic domains. Whereas the functions of these additional UBP domains are not well understood, recent studies have suggested that they function in substrate recognition, cofactor association (15, 16), and subcellular localization (17, 18).

Whereas the human herpesvirus-associated USP has been shown to be necessary and sufficient for deubiquitination of its specific substrate, the p53 tumor suppressor (19), other characterized UBPs have been shown to require additional protein cofactors for activity. For example, USP10, one of the human UBPs, required the Ras-GAP Src homology 3-binding protein,
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G3BP1, for catalytic activity (20). Ubp3, the yeast homologue of human USP10, has been shown to form a complex with the Bre5 cofactor to specifically deubiquitinate the Sec23 (21) and \( \beta^-\)COP (22) subunits of the COPII and COPI complexes that regulate anterograde and retrograde transport between the endoplasmic reticulum and the Golgi apparatus (21), respectively. Further analysis of the Ubp3-Bre5 complex reveals that Bre5 is an essential cofactor for Ubp3-mediated catalysis (21). Taken together, it appears that Bre5 is a key component of Ubp3-mediated function, although the molecular basis for this is currently not known.

Yeast Bre5 has two recognizable domains, a nuclear transfer factor 2 (NTF2) domain at the N terminus (residues 8–140) and an RNA recognition motif (RRM) at the C terminus (residues 419–481), and Ubp3 contains no recognizable domains outside of its C-terminal catalytic domain. Although NTF2-like domains are found in many proteins with diverse functions, (23–26), a common function of these domains appears to be that they mediate protein-protein interactions. Indeed, deletion analysis of Bre5 reveals that its NTF2 domain, but not the RNA-binding domain, is necessary and sufficient to bind Ubp3 and is required for Ubp3 function in vivo (21, 22), suggesting that the NTF2 domain of Bre5 may directly associate with Ubp3 to regulate its deubiquitination activity.

The NTF2-like domains from NTF2 (23, 24, 27, 28), TAP and P15 (29), and Mtr2 (25) and Mex67 (26) alone and, in some cases, in complex with their cognate protein targets have been characterized at both the biochemical and structural levels. These studies reveal that this domain forms homo- or heterodimers with low micromolar affinity (30) and that the NTF2-like dimers bind protein targets, some of which contain FXFG or FG repeats. Interestingly, although the various NTF2-like domain structures have similar overall dimeric folds, the region of the NTF2-like domain that is used for recognition of these repeats is not conserved among the different proteins, possibly reflecting the relatively low primary sequence homology between the NTF2-like domains (25) and their diverse biological roles (26). Indeed, the observation that Ubp3 does not contain FXFG or FG repeats within its primary sequence suggests that its mode of recognition by the NTF2-like domain of Bre5 may also be novel.

In this study, we employ biochemical and structural analysis to show that the NTF2-like domain of Bre5 forms a tight homodimer that directly associates with a folded domain within the N terminus of Ubp3. We also use the crystal structure of the Bre5 NTF2-like domain to inform structure-based mutagenesis to further characterize the molecular basis for the Bre5-Ubp3 interaction in vitro and show the importance of this interaction in vivo. Together, these studies provide new insights into protein recognition by NTF2-like domains and provide a molecular scaffold for understanding how Ubp3 function is regulated by Bre5 cofactor binding.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—The UBP3 gene was amplified by PCR from yeast genomic DNA and inserted into the pGEX4T expression vector for further subcloning. Ubp3 fragments encoding residues 1–912 (full-length), 50–912, 104–912, 189–912, and 260–912 were subcloned into the PET28a expression vector as C-terminal His\(_{6}\)-tagged fusion proteins. DNA encoding residues 1–146 of Bre5 was PCR-amplified from pETM116 and cloned into the pGEX4T-1 expression vector for the preparation of the N-terminal GST fusion protein.

All Ubp3 and Bre5 expression plasmids were transformed into Escherichia coli strain BL21 (DE3) for protein expression. Transformed bacteria were initially grown at 37 °C to an absorbance of 0.7–0.9 at 600 nm, and protein overexpression induced by addition of 0.1 mM isopropyl-1-thio-\( \beta\)-D-galactopyranoside followed by overnight growth at 15 °C. Cells were disrupted by sonication in a solution containing PBS buffer supplemented with 10 mM \( \beta\)-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. For the purification of His\(_{10}\)-tagged fusion protein, the protein was partially purified using an Ni\(^{2+}\)-NTA resin as described by the manufacturer and further purified using anion exchange (Q-Sepharose) and gel filtration (Superdex-200) in PBS buffer. For purification of GST-tagged proteins, the supernatant was partially purified using glutathione resin (Novagen) as described by the manufacturer.

Unfused Bre5-(1–146) was prepared by treating a slurry of the resin-bound GST fusion proteins with thrombin (10 units/mg fusion protein) overnight at 4 °C, and the unfused Bre5 protein was eluted with PBS buffer. Bre5-(1–146) was further purified with gel filtration (Superdex-75) in PBS buffer. Protein purity was judged to be greater than 90% by SDS-PAGE, and the protein was concentrated to 10–20 mg/ml in PBS for storage at −70 °C. Selenomethionine-derivatized Bre5-(1–146) was overexpressed from pGEX4T-Bre5-(1–146) transformed bacterial strain BL21(DE3) (Novagen) and grown in MOPS-based minimal medium. Selenomethionine-derivatized protein was purified and stored essentially as described for the underivatized protein.

All site-directed Bre5-(1–146) mutations were prepared with the appropriate primers and the QuikChange mutagenesis kit essentially as described by the manufacturer (Stratagene), and the mutant proteins were purified essentially as described for the wild-type protein. The secondary structure of Bre5 protein fragments was analyzed using circular dichroism on a 62A DS spectropolarimeter (Aviv Associates, Lakewood, NJ) by using 300 \( \mu \)l of the respective sample at 10 °C. The protein concentration was about 30 \( \mu \)M in a buffer containing 20 mM Hapes, pH 7.0, 100 mM NaCl, and 10 mM \( \beta\)-mercaptoethanol.

**Sedimentation Equilibrium Analysis of Bre5**—The oligomerization properties of full-length Bre5-(1–146) and the C117A mutant were analyzed using sedimentation equilibrium on a Beckman XL-1 analytical ultracentrifuge in a buffer containing 20 mM HEPES, pH 7.0, and 100 mM NaCl. Three starting protein concentrations (0.5, 0.75, and 1.0 mg/ml) and three centrifugation speeds (22,000, 30,000, and 40,000 rpm) were used with absorption optics for analysis. After equilibrium at each speed, as assessed by comparison of absorbance optics using the MATCH program for data editing was performed using the REDFIT program (both programs were provided by the National Analytical Ultracentrifugation Facility, Storrs, CT).

The NONLIN program (31) was used to globally fit several scans for each set of experiments. NONLIN fits used an effective reduced molecular weight of \( \sigma \). For models of associating systems, \( \sigma \) was held at the correct value based on the known monomer molecular weight of the protein. The equilibrium constants were fitted as \( \ln K \). These values were converted to dissociation constants with the appropriate molar units. The fit quality of the models for all of the experiments was determined by examination of residuals and by minimization of the fit variance.

**In Vivo Bre5-Ubp3 Binding Studies**—For pull-down assays, GST-Bre5-(1–146) protein (50 \( \mu \)M in PBS buffer) was incubated with purified His\(_{6}\)-Ubp3 deletion constructs (50 \( \mu \)M in PBS buffer) for 1 h at 4 °C, followed by immobilization on 30 \( \mu \)l of glutathione-Sepharose beads for 1 h at 4 °C. Beads were washed three times with 1 ml of PBS buffer, and 10-\( \mu \)l aliquots were mixed with an equal volume of 2× SDS-loading buffer and analyzed on SDS-PAGE.

All isothermal titration calorimetry measurements were carried out using a MicroCal VP-ITC isothermal titration calorimeter (MicroCal, Inc.), and each experiment was carried out in duplicate. Untagged Ubp3-(181–282) protein was diluted to 50 \( \mu \)M in PBS buffer and added to the 1.4-mM sample cell, and a 0.3–0.5 mM solution of wild-type or mutant Bre5-(1–146) protein titrant was loaded into the injection syringe. For each titration experiment, a 60-s delay at the start of the experiment was followed by 35 injections of 10 \( \mu \)l of the titrant solution. The sample cell was stirred at 300 rpm throughout and maintained at 15 °C. Titration data were analyzed using the Origin 5.0 software supplied by MicroCal Inc., and data sets were corrected for base-line heats of dilutions from control runs as appropriate. The corrected data were then fit to a theoretical titration curve describing one binding site per titrant. The area under each peak was integrated and plotted against the molar ratio of Ubp3 to Bre5. A nonlinear best fit binding isotherm for the data was used to calculate Ubp3/Bre5 stoichiometry, dissociation constant, and standard enthalpy change.

**In Vivo Sec23 Deubiquitination and Growth Assays**—Yeast cultures were grown either in rich medium (YPD; Q-Biogen) or in minimal medium (SD) containing 0.67% yeast nitrogen base with ammonium sulfate, 2% dextrose, and supplemented with appropriate nutrients.
(32). Yeast transformations were performed with standard procedures and preparation of the BY45a strain as previously described (22).

The preparation of fusion proteins between Bre5 or its truncated version, the LexA DNA binding domain expressed from the pBTM116 plasmid, has been previously described (22). The point mutations in Bre5-His<sub>1423</sub>, Bre5-His<sub>1338</sub>, and Bre5-His<sub>1423</sub> as well as the C-terminal His tag were introduced by PCR, and the resulting products were cloned into BamHI/Xhol sites of the P426-ADH plasmid (33).

Yeast cells grown in YPD or minimal medium were collected during the exponential growth phase (A<sub>600</sub> = 2 or 0.8, respectively). Total protein extracts were prepared by the NaOH-TCA lysis method (21). Protein samples were separated by 7% SDS-PAGE, transferred to nitrocellulose membranes, probed with appropriate antibodies, and detected with chemiluminescence protein immunoblotting reagents (Pierce). Rabbit polyclonal antibodies to Sec23p (1:400 dilution) were kindly provided by B. Lesch and R. Schekman, and mouse monoclonal antibody anti-His and rabbit polyclonal antibody anti-Lex were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Invitrogen, respectively.

Growth assays of vector-transformed cells at different pH values were carried out as described above except that parallel plates were carried out at pH 6.5 and 8.0, and growth was monitored by cell plating.

Crystalization and Structure Determination of Bre5-(1–146)—Crystals of Bre5-(1–146) were grown at room temperature using the hanging drop vapor diffusion method. 2 μl of protein solution at 14 mg/ml (0.87 mM) in buffer (20 mM HEPES, pH 7.0, 5 mM DTT, and 100 mM NaCl) was mixed with an equal volume of reservoir solution containing 25% polyethylene glycol 3350, 100 mM sodium citrate, pH 5.5, 0.25 M ammonium sulfate and equilibrating over 0.5 ml of reservoir solution. Crystals grew to a typical size of 200 × 100 × 100 μm over 3 days and were flash frozen in a reservoir solution supplemented with 20% glycerol for storage in solid propane prior to data collection.

Single wavelength native and three-wavelength (peak, inflection, and remote) multiple anomalous diffraction data for selenium-derivatized crystals were obtained on beamline X25 at the Brookhaven National Laboratories using an ADSC Quantum-4 CCD detector at 100 K. The diffraction data and processed with the HKL Research Inc., and the relevant statistics are summarized in Table I. Three selenium sites were identified using CNS and SOLVE (34, 35). Phases were combined using CNS, and the resulting experimental electron density map was improved by solvent flipping density modification. The program O (36) was used to build the model of the protein using the selenium methionine positions as guides. Model refinement employed simulated annealing and torsion angle dynamic refinement protocols in CNS with iterative manual adjustments of the model using the program O, with reference to 2Fo – Fc and Fc – Fo electron density maps. Toward the later stages of refinement, individual atomic B-factors were adjusted, and solvent molecules were modeled into the electron density map. The final model was checked for errors with a composite-simulated annealing omit map. A final round of refinement resulted in a model with good refinement statistics and geometry (Table I).

RESULTS

Mapping and Characterization of the Bre5 Binding Site in Ubp3—We employed a GST-Bre5 fusion protein harboring the NTF2-like domain of Bre5 (GST-Bre5-(1–146)) and His<sub>6</sub>-tagged Ubp3 fusion proteins to map theBre5 binding site on Ubp3. Initial pull-down experiments (data not shown) revealed that GST-Bre5-(1–146) did not show detectable interaction with the C-terminal catalytic domain of Ubp3 (residues 389–912). Based on this, we tested whether the N-terminal region of Ubp3 mediated Bre5 interaction. To address this possibility, we prepared a series of N-terminal Ubp3 deletion constructs (deleting about 50 residues at a time) for GST pull-down experiments. Specifically, we prepared His<sub>6</sub> fusion proteins containing Ubp3-(1–912), (50–912), (150–912), (150–912), and (260–912) (Fig. 1a). As can be seen in Fig. 1a, each of the Ubp3 deletion constructs, except for Ubp3-(260–912), were pulled down with GST-Bre5-(1–146) at levels comparable with the intact protein. The His-Ubp3-(260–912) deletion construct did not show any pull-down with GST-Bre5-(1–146). These experiments revealed that a region within residues 189–260 of Ubp3 is necessary for interaction with the Bre5 NTF2-like domain.

A secondary structure prediction of Ubp3 using PSIpred reveals two contiguous regions of high secondary structure, residues 280–912, roughly corresponding to the Ubp3 catalytic domain, and residues 186–272, roughly corresponding to the region of Ubp3 that we identified is involved in Bre5 interaction. Taken together, our studies suggested that residues 186–272 of Ubp3 constitute a folded protein domain that mediates Bre5 interaction. In order to directly test this hypothesis, we prepared two new recombinant Ubp3 constructs in which GST was fused to either residues 181–260 (GST-Ubp3-(181–260)) or residues 181–282 (GST-Ubp3-(181–282)). These GST fusion proteins were overexpressed in E. coli and purified by GST affinity, followed by thrombin cleavage to remove the GST component. In agreement with secondary structure predictions, a CD spectrum of these Ubp3 domains was consistent with the presence of a mainly helical protein domain (Fig. 1c).

To determine whether recombinant Ubp3-(181–282) was sufficient for interaction with the NTF2-like domain of Bre5, we carried out a series of additional biochemical experiments. We first prepared a 2:1 stoichiometric complex of Bre5-(1–146) and Ubp3-(181–282) (since we expected an NTF2-like domain dimer to bind one Ubp3 subunit) and subjected the complex to gel filtration analysis. As can be seen from Fig. 1d, the two protein fragments coelute as a homogeneous complex. GST-pull-down studies employing GST-Bre5-(1–146) and untagged Ubp3-(181–260) and Ubp3-(181–282) also showed that the Ubp3 N-terminal domains bound to Bre5 as efficiently as full-length Ubp3 (Fig. 1e). These studies suggest that a region within residues 181–260 of Ubp3 is necessary and sufficient for interaction with the NTF2-like domain of Bre5.

We recently reported that Bre5 and Ubp3 forms an active deubiquitination complex that cleaves off ubiquitin from Sec23, a COPII subunit essential for the transport between the endoplasmic reticulum and the Golgi apparatus (22). In particular, absence of the Bre5p-Ubp3p complex induces an accumulation of monoubiquitylated Sec23 that facilitates its subsequent polyubiquitination and degradation by the 26 S proteasome. To test whether the interaction between the NTF2-like domain of Bre5 and Ubp3 is sufficient to regulate the deubiquitination of Sec23 in vivo, we analyzed Bre5 deletion cells transformed with a plasmid encoding for fusion proteins between LexA and either wild-type Bre5 (Lex-Bre5), the NTF2-like domain of Bre5p (Lex-Bre5-(1–145)), or Bre5 deleted in the NTF2-like domain (Lex-Bre5-(145–515)). As shown in Fig. 1e, plasmid-encoded LexA-Bre5 was able to restore deubiquitination of Sec23, whereas Lex-Bre5-(1–145) showed a modest level of complementation. As expected from previous data (21), similar amounts of Lex-Bre5-(145–515) did not complement the Bre5 deletion. Taken together, with in vivo binding studies, these results indicate that whereas the NTF2-like domain of Bre5 is sufficient to promote interaction with Ubp3, the intact Bre5 protein is required to fully facilitate Ubp3-mediated deubiquitination of Sec23 in vivo.

Overall Structure of NTF2-like Domain of Bre5—To obtain more mechanistic insights into how the NTF2-like domain of Bre5 may mediate Ubp3 interaction, we crystallized the NTF2-like domain of Bre5 for x-ray structure determination. Recombinant Bre5-(1–146), in which the N-terminal GST fusion was cleaved with thrombin, eluted between the 44- and 17-kDa globular protein standards upon gel filtration (Fig. 1d), suggesting that the protein was dimeric in solution, consistent with the structures of NTF2-like domains from other proteins (30). Crystals of the Bre5 NTF2-like domain were obtained in the space group P6<sub>1</sub>22 with two molecules per asymmetric unit cell, and the structure was determined using multiple anomalous
lous diffraction with anomalous signals from three ordered selenium ions from selenium methionine-derivatized protein, and the structure was refined to 2.1 Å resolution with excellent refinement statistics and geometry (Table I).

The NTF2-like domain of Bre5 adopts a mixed α/β fold that is similar to other NTF2-like domain structures (Fig. 2a). Briefly, each subunit of Bre5-(1–146) contains a six-stranded (β1–β6) anti-parallel β sheet that forms a curved platform for three α helices (α1–α3) that lie on the concave side of the β-sheet (Fig. 2a). The two subunits of the dimer in the asymmetric unit cell are almost identical, with a root mean square deviation of 1.1 Å for all atoms. The most significant differences between the two molecules map to residues 107–113 and 125–130 that form the β4-β5 and β5-β6 connecting loops, respectively. In addition, residues 43–54 of the β1-β2 loop are not modeled in either protein subunit of the protein dimer and are presumed to be disordered. Superposition of the Bre5 NTF2-like domain with the structures of other nascent NTF2-like domains from yeast Mex67 (1OF5), rat NTF2 (1OUN), yeast NTF2 (with bound Ran GDP; 1GYB), rat NTF2 (with bound FXFG repeat peptide; 1A2K), and human TAP/P15 (with FG repeat peptide from nucleoporin; 1JN5) have root mean square deviations of 1.4, 1.1, 1.2, 1.1, and 1.2 Å for Ca atoms, respectively, reinforcing the high degree of structural homology.

**Fig. 1.** Characterization of the Bre5 binding domain of Ubp3. A, schematic diagram illustrating the truncations of Ubp3 used in the in vitro GST pull-down assays. The gray bar depicts the N-terminal region of Ubp3 shown in this study to bind Bre5, and the black bar depicts the catalytic domain of Ubp3. B, results of the GST pull-down assays using GST-Bre5(1–146) and deletion fragments of Ubp3. C, CD spectra of Ubp3 protein fragments. D, purification of a Bre5-Ubp3 complex on Superdex 75a gel filtration using Bre5-(1–146) and Ubp3-(181–282). An SDS-polyacrylamide gel of fractions from the main peak of the complex is also shown. E, SDS-PAGE analysis and Western blotting of extracts from Bre5Δ cells transformed with vector expressing different LexA-Bre5 fusion proteins. The Western blotting employed antibodies against ubiquitinated Sec23 (upper panel) or LexA (lower panel).
within the NTF2-like domains, despite the relatively low sequence conservation (data not shown).

Also, like other NTF2-like structures, the Bre5 NTF2-like domain forms an analogous dimer with a root mean square deviation of the dimers between 2 and 3 Å for Cu atoms (Fig. 2b). The dimer interface appears somewhat more extensive for Bre5 than other NTF2-like domain dimers, burying about 2478 Å² of solvent-excluded surface, which represents about 30% of the surface area of each of the protein subunits. Briefly, the convex surfaces of the β-sheets from each of the subunits face each other such that residues from β3 and β6–β8 mediate all of the dimer contacts (Fig. 2c). Together, the dimer is stabilized by 15 hydrogen bonds formed by residues Glu36, Thr38, Asp87, Phe88, Gln89, Tyr90, Asn135, and Asp136 as well as several residues that mediate van der Waals interactions, including Thr236, Thr229, Leu269, His268, Met101, Thr103, Cys117, Ile121, Ile127, and Ile144. Notably, almost all of the residues that stabilize the dimer map to residues that are highly conserved within the strands β3–β6 of putative Bre5 homologues from other strains of yeast and other species (Fig. 2e), suggesting that these homologues adopt similar dimeric structures.

An interesting aspect of the Bre5 dimer in the crystals is that the electron density map reveals that Cys117 of one subunit of the dimer (chain B) adopts two conformers, with one of the conformers in position to form a disulfide bond across the dimer interface with Cys117 of the opposing subunit of the dimer (chain A) (Fig. 2d). An occupancy refinement suggests that 20% of the dimers are disulfide-linked (with good stereochemistry) within the NTF2-like domain of Bre5 forms a tight dimer and that a Cys117-mediated disulfide contributes to the stability of this dimer under nonreducing conditions. In contrast, a similar analysis of the Bre5-(1–146)C117A mutant produced a good fit for a monomer-dimer equilibrium model with a dissociation constant of about 245 nM (Fig. 3b). Together, these data reveal that the NTF2-like domain of Bre5 forms a tight dimer and that a Cys117-mediated disulfide contributes to the stability of this dimer under nonreducing conditions.

The midnanomolar dissociation constant for the non-disulfide-linked NTF2-like domain of Bre5 is in contrast to the significantly weaker dissociation constant of about 1 μM for the NTF2 dimer, which has led to the proposal that a substantial portion of cellular NTF2 might be monomeric (30) In contrast, our data suggest that in vivo a substantial fraction of Bre5 is dimeric, and the portion of Bre5 that may be associated with the endoplasmic reticulum, where disulfide bonds can occur (37), is exclusively dimeric. In light of this, it is possible that disulfide bond formation within the NTF2-like domain of Bre5 might modulate dimer formation for the regulation of Bre5 and Ubp3 activity in vivo.
Molecular Dissection of the Ubp3-Bre5 Interaction—In order to probe the binding surface of the NTF2-like dimer of Bre5 for Ubp3 binding, we used the structure of Bre5 as a scaffold for site-directed mutagenesis. The structure of Bre5 reveals three surface patches that contain grooves as potential sites for interaction. The first patch lies between the β3-β4 and β5-α3 loops, close to one edge of the dimer interface (patch 1 in Fig. 4a); the second patch lies between the β3-β4 and α2-β3 loops and the exposed surface of the α3 helix of each of the subunits (patch 2 in Fig. 4a); and the third patch lies between the β4-β5
loop, the C terminus, and the disordered $\beta_1-\beta_4$ loop near the edge of the dimer interface opposite to patch 1 (patch 3 in Fig. 4a). In order to probe the potential importance of these Bre5 protein surface patches for Ubp3 binding, we chose, for mutagenesis, a subset of surface-exposed residues in these three regions that were either conserved in the Bre5 homologues from yeast or conserved within the putative Bre5 homologues from other species (Fig. 2e). The patch 1 mutants included L94E, L94K, P124L, and F131K; the patch 2 mutants included Q14L, F30A, and F30D; and the patch 3 mutants included Y42D and R139F. Each of these mutants was prepared in the context of the GST-Bre5-(1–146) fusion protein, purified to homogeneity, and assessed for its relative ability to bind Ubp3-(189–912) using GST pull-down assays. As can be seen in Fig. 4b, Bre5 mutants in patches 1 and 2 show no detectable effect on Ubp3 association, whereas both mutations within patch 3 do show reduced Ubp3 association. In light of this result, we prepared the Y42D/R139F Bre5 double mutant, and as can be seen in Fig. 4b, this double mutant shows even more reduced binding to Ubp3. To confirm that the Bre5-(1–146) patch 3 mutation proteins (Y42D, R139F, and Y42D/R139F) did not perturb the overall structure, we used CD to confirm that the secondary structure was not disrupted (data not shown).

To further quantitate the binding between Ubp3 and Bre5 and the patch 3 Bre5 mutants, we performed isothermal titration calorimetry studies employing the recombinant Ubp3-(181–282) and Bre5-(1–146) protein constructs. We first titrated wild-type Bre5-(1–146) into a solution containing Ubp3-(181–282). As illustrated in Fig. 5a, this experiment provided excellent data that fit well to a model in which one Ubp3 subunit binds a Bre5 dimer with a dissociation constant of about 187 nM. Similar analysis employing the Y42D, R139F, and Y42D/R139F Bre5 mutant proteins yielded similar stoichiometry but higher dissociation constants of about 299 nM, 937.6 nM, and 10 $\mu$M, respectively (Fig. 5, b–d). The nearly 60-fold increase in dissociation constant for the Y42D/R139F Bre5 mutant suggests that the patch 3 binding groove of Bre5 is used in Ubp3 association. Moreover, the stoichiometry measurements for the complexes reveal that a Bre5 dimer binds one subunit of Ubp3. To further assess the importance of the Bre5 dimer for Ubp3 association, we carried out analogous isothermal titration calorimetry studies employing the Bre5-(1–146)C117A mutant, since we showed earlier that Cys117 mediates Bre5 dimer formation. As illustrated in Fig. 5e, Bre5-(1–146)C117A binds Ubp3 with a dissociation constant of about 555 nM, about 3-fold higher than native Bre5-(1–146). This finding is consistent with the importance of Bre5 dimer formation for Ubp3 binding.

To test the importance of Tyr42 and Arg139 of the patch 3 region of Bre5 for Ubp3 interaction in vivo, we compared the ability of the wild type and Bre5 mutants to mediate Sec23 deubiquitination in cells. For these studies, Bre5Δ cells were transformed with a plasmid encoding either wild type Bre5p (Bre5-His) or a Bre5 mutant encoding the Y42D (Bre5-His$_{Y42D}$) or Y42D/R139D (Bre5-His$_{Y42D/R139D}$) mutations as His-tagged
Figure 5. Characterization of Bre5/Ubp3 interactions. A–E, binding isotherms for isothermal titration calorimetry runs in which wild-type or mutant Bre5-(1–146) is titrated into a solution containing Ubp3-(181–282). Data from one of the duplicate runs is shown. The area under each injection spike (above) is integrated and fitted using nonlinear least squares regression analysis (below). The calculated dissociation constant ($K_d$) and stoichiometry ($N$) are indicated below the respective curve. F, SDS-PAGE analysis and Western blotting of extracts from Bre5Δ cells transformed with vector expressing His-tagged versions of wild-type Bre5 (Bre5-His), Bre5 in which the residue Tyr42 has been mutated to Asp (Bre5-HisY42D), Bre5p in which the residue Arg139 has been mutated to Asp (Bre5-HisR139D), or both (Bre5-HisY42D/R139D). The Western blotting employed antibodies against ubiquitinated Sec23 (upper panel) or His (lower panel). G, growth assay for Bre5 cells, Bre5Δ cells, or Bre5Δ cells transformed with vector expressing either Bre5-His or Bre5-HisY42D/R139D and grown at either pH 6.5 (left panel) or pH 8.0 (right panel).
fusion protein. The ability of these proteins to interact with Ubp3 and therefore to complement Bre5 deletion was measured by following the deubiquitination of Sec23. As shown in Fig. 5f, whereas a single Y42D mutation does not show a detectable effect on the deubiquitination of Sec23, the Y42D/R139D double mutant resulted in detectable levels of ubiquitinated Sec23. Since disruption of Bre5 or Ubp3 has also been shown to confer hypersensitivity to cell growth at basic pH (38), we also assayed the relative growth sensitivity of the wild-type and Y42D/R139D double mutant upon exposure to basic pH. As can be seen in Fig. 5g, the double mutant shows a modest but reproducible defect in cell growth. Taken together, these in vivo studies reinforce the importance of residues Tyr(24), Tyr(32), Ile(65), Phe(115), Gln(118), and Phe(210) conserved roles in the folding of the NTF2 monomer (Fig. 2c). In contrast, residues that mediate dimer formation and residues located at the dimer surface do not show conservation. In light of this, it is perhaps not surprising that structures of various NTF2-like domains in complex with their cognate proteins have revealed disparate modes of substrate recognition. For example, the structure of the TAP/P15 NTF2-like heterodimer bound to an FG repeat peptide from nucleoporin (23, 29) reveals that the FG sequence interacts with a TAP groove analogous to patch 1 of Bre5, and the structure of the NTF2 homodimer bound to FXFG repeats reveals a similar interaction surface (23, 29, 39, 40). In contrast, the structure of the NTF2 homodimer bound to the Ran GTPase reveals that the Ran GTPase makes specific and non-specific interactions with NTF2 in grooves analogous to patches 2 and 3 of Bre5. Strikingly, in each of these NTF2 complexes, the binding stoichiometry is one subunit of NTF2-like domain to one subunit of respective protein target (41–43).

Our studies of the interaction between the NTF2-like domain of Bre5 and Ubp3 reveal yet another mode of protein recognition by NTF2-like domains. Although the interaction between Bre5 and Ubp3 appears to involve a similar region (patch 3) that is employed for specific Ran GTPase interaction with NTF2, the binding stoichiometry for the Bre5-Ubp3 complex is 2:1 as opposed to 1:1 for the NTF2-Ran complex. This observation suggests that, unlike the NTF2/Ran GTPase interaction and other NTF2-like domain-mediated interactions, Ubp3 binding may take place at a dimer-induced site and possibly across the Bre5 dimer interface. In addition, the dissociation constant that we measured for the Bre5/Ubp3 of 187 nM is unusually high relative to other reported protein-protein interactions (44), also highlighting a significant difference. Indeed, it appears that the relatively compact NTF2-like domain dimer has evolved a number of different modalities for protein-specific recognition.

Although our studies have focused on the mode of interaction between the Bre5 NTF2-like domain and Ubp3 and not on the mechanism of how the NTF2-like domain of Bre5 mediates Ubp3 deubiquitination activity, our studies do provide testable models for how Bre5 might regulate Ubp3 activity. The Bre5 NTF2-like domain might regulate Ubp3 activity by contacting the Ubp3 catalytic domain (located within the Ubp3 C terminus) or regulating the appropriate interaction with Ubp3 substrates. Alternatively, the Bre5 NTF2-like domain might relieve Ubp3 repression, either by another protein interacting with Ubp3 or its substrate or by another region of Ubp3, possibly the N-terminal residues 181–260 of Ubp3. Our previous studies indicate that Bre5 is not required for the direct recognition of the unmodified Ubp3 substrate, Sec23, in vitro (21). Our studies also rule out the direct interaction between the Bre5 NTF2-like domain and the catalytic domain of Ubp3 to either activate or repress Ubp3 activity, since our pull-down studies show that residues 181–260 of Ubp3 are necessary and sufficient for BRE5 NTF2-like domain interaction (Fig. 1b) and since we cannot detect binding between the Ubp3 N terminus and C-terminal catalytic domain in trans using pull-down studies or in two-hybrid assays performed in wild-type cells and cells disrupted for Bre5 (data not shown). Our in vitro results also demonstrate that the N-terminal NTF2-like region of Bre5 alone cannot fully complement a Bre5 deletion for Ubp3-mediated deubiquitination of Sec23 (Fig. 1e), suggesting that the N-terminal NTF2-like region and the remaining C-terminal region of Bre5 cooperate for Ubp3-mediated ubiquitination. Given these results, we propose that Bre5 facilitates the access of Ubp3 to its ubiquitylated substrate in vitro, either by contacting the ubiquitylated Sec23 substrate in an ubiquitin-dependent manner or by binding and releasing an Ubp3 inhibitor protein bound to the modified form of the Ubp3 substrate. It is attractive to propose that this Bre5-mediated recognition might involve patch 1 and/or 3 of the Bre5 NTF2-like domain, although structural analysis of a relevant Bre5-Ubp3 complex will be required to more directly address this possibility.

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