SUPPORTING INFORMATION

Deubiquitinase Ubp3 enhances the proteasomal degradation of key enzymes in sterol homeostasis

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CONTENTS

Supplementary methods

Yeast strains, plasmids and growth conditions

Strains, plasmids and primers used in this study are list in Table S1-S2. All yeast strains are derivatives of the wild-type strain JMP024 (4741 background). For phenotype experiments, all 21 endogenous DUB genes except MIY1 and RPN11 were deleted by PCR-based homologous recombination and substituted by a G418-resisitant gene, respectively (1). To analysis the protein expression level of Erg1 and Erg3 we labeled them with 6 x Histidine and streptavidin (HB) tags and expressed on a 2μ plasmid (pUB221) by Gibson reactions, respectively (2). A consistent tag coupled Ubp3 was constructed as well, the catalytic activation site C469A mutation plasmids of Ubp3 was constructed by site-directed mutagenesis kit. In addition, a plasmid expressed free HB was used as control to deduct the background when enriching their interactors. To construct these plasmid-expressed strains (G418-resisitant), ubp3 was replaced with a nourseothricin gene from pDB213 plasmid. These plasmids were transformed into WT or *ubp3*Δ (nourseothricin-resistent) strains as indicated. Standard genetic techniques were used. Unless otherwise stated, yeast strains were grown at 30 °C and cultured in YPD (1% yeast extract, 2% peptone and 2% dextrose) or complete synthetic medium (0.67% yeast nitrogen base with ammonium sulfate, 2% glucose and supplemented with the appropriate amino acids, SC) and harvested when OD_{600} up to 1.5 unless indicated.

To confirmed whether or not the protein was degraded in a proteasome-dependent manner, 50 μM PS341 (Selleck Chemicals, Houston, Texas, USA) was added into SC media on the condition that OD_{600}=0.8 and continued culture at 30 ℃ for 4 hours before harvested. To analysis the protein degradation in the presence of fluconazole, 50 μM PS341 was added into SC media on the condition that OD_{600}=0.8 and continued culture at 30 ℃ for 0.5 hours before stimulated with 32μg/mL fluconazole (National Institutes for Food and Drug Control, Beijing, China) for 4 hours, and harvested.

Susceptibility of strains to antifungal agents

To test the response of yeast strains to several kinds of antifungal agents (terbinaifine and

amphotericin B (National Institutes for Food and Drug Control, Beijing, China), liranaftate (Toronto Research Chemicals, Toronto, Canada)), yeast cells were incubated in YPD until growth to exponential phase. Cells corresponding to 2 OD of yeast cells were collected at 3000g for 3 minutes at room temperature, and washed three times with sterile water. Serial dilution of 1 OD cells with sterile water to gain 10^7 cells per mL, 10^6 cells per mL, 10^5 cells per mL and 10^4 cells per mL suspension. 2 μL suspension of each sample was spotted on plates of SC or SC containing antifungal agents. The plates were incubated for about 72 hours at 30 ℃ and paragraphed.

The IC_{50} of fluconazole to yeast cell were measured in SC medium as described (3). A single colony of *S. cerevisiae* was resuspended in distilled water and washed three times. The washed cells were inoculated into the 200 μL SC media containing gradient dilution of fluconazole (256, 128, 64, 32, 16, 8, 4, 2, 0 μg/ mL) at the OD_{600}=0.05 in 24-well plates, measured the turbidity of each well after 12h of incubation in 30℃ at 220 rpm. The OD_{600} of each strain in individual fluconazole concentrations were measured on triple biological replicates. The IC_{50} of each strain was calculated by software GraphPad Prim 5 (GraphPad Prism, San Di-ego, CA, USA).

SILAC labeling for quantitative proteomics

For SILAC experiments, lysine and arginine auxotrophic strains were metabolically labeled in SC light medium containing 30 mg/mL lysine and 20 mg/mL arginine or heavy medium with 30 mg/mL $^{13}C_6$-lysine and 20 mg/mL $^{13}C_6{}^{15}N_4$ arginine (Cambridge Isotope Laboratories, Andover, MA, USA). Half of the cells were harvested at early logarithmic phase (OD_{600}=1.0), and the remaining cells were incubated with 32 μg/mL fluconazole for 4 hours before collection. Equal amounts of light and heavy isotope-labeled cells were mixed immediately before centrifugation and stored at -80 ℃.

Protein extraction and sample preparation for LC-MS/MS analysis

Yeast cells were splitting decomposition by glass beads disruption in denatured lysis buffer (8 M urea, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 50 mM iodoacetamide (IAM), 1 mM Phenylmethanesulfonyl fluoride (PMSF) and 1 mM N-ethylmaleimide (NEM)) for 5 x 20 s in a Bertin precellys™ 24 homogenizer (Bertin Technologies, France) with 1 min breaks on ice

between runs. The mixture was subjected to centrifugation at 21000 g at 4 ℃. Total cell lysates (~120 μg) in supernatant were resolved on a 10% SDS-Polyacrylamide gels and cut into slices based on the molecular weight marker and the protein content, respectively. The protein in each gel band was in-gel digested with trypsin overnight. The tryptic peptides were extracted from gels with extraction buffer (5% formic acid (FA) and 50% acetonitrile (ACN)) and then pure ACN and finally dried using a vacuum dryer (Labconco CentriVap, Kansas City, MO, USA) before LC-MS/MS analysis.

LC-MS/MS analysis

Peptides were analyzed by an ultra-performance LC-MS/MS platform of hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Waters nano-ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). We employed a 75 μm i.d. × 15 cm capillary column (Beijing SpectraPeaks, Beijing, China) packed with 3 μm C18 reverse-phase fused silica (Michrom Bioresources, Inc., Auburn, CA, USA) to resolve our peptide mixtures based on polarity of the peptides and eluted gradually with a 60 min nonlinear gradient ramped from 8–40% of mobile phase B (phase B: 0.1% FA in ACN, phase A: 0.1% FA and 2% ACN in water) at a flow rate of 0.3 μL/ min. Eluting peptides were analyzed using the mass spectrometer as described before (4). The MS1 precursor was analyzed with a mass range of 350–1,800 at a resolution of 30,000 at m/z = 400. The automatic gain control (AGC) was set at 1×10^6 and the maximum injection time (MIT) was set at 150 ms. MS2 spectra were collected in data-dependent mode for the 10 most intense ions, which were subjected to fragmentation via collision induced dissociation (CID) with 35% normalized collision energy in the LTQ. For each scan, the AGC was set at 1×10^4 and the maximum injection time at 25 ms. The dynamic range was set at 30–60 s to suppress repeated fragmentation of the same peaks.

Data analysis

All the raw files were searched by MaxQuant (version 1.5.30) against the target-decoy protein FASTA file from Saccharomyces Genome Database (version released in 2016.02, 6717 entries) along with 112 common contaminants (ftp.thegpm.org/fasta/cRAP). The decoy components were

constructed by pseudo-reversed sequences of all target proteins (5,6). For protein quality, specific digestion of trypsin was used with a maximum of two missed cleavages allowed. The precursor mass tolerance was 20 ppm, and the MS/MS mass tolerance was 0.5 Da. Carbamidomethylation of cysteine was set as a fix modification and oxidation of methionine was assigned as a variable modification. A minimal peptide length of seven amino acids was required. The FDR < 1% on spectrum, peptide and protein level was used as filtering criteria. For the SILAC experiment, $^{13}C_6$ of lysine (K6, +6.0201 Da) and $^{13}C_6$ $^{15}N_4$ of arginine (R10, +10.0083 Da) were added as heavy label. For comparison of the protein abundance in SILAC label-swap experiments, fold change and *p*-value were both used to calculate the significantly changed proteins in ubp3 knockout strain. Only proteins with more than 1.4 folds change and *p*-value < 0.05 both in forward and reverse experiments were considered as significantly changed proteins.

TAP strategy used to enrich protein and its interactors

6 x His and streptavidin double-tagged (HB) fusion proteins were purified from cell lysates by tandem affinity purification (TAP) procedures (7). Yeast cells were lysed with glass beads in native condition (50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 10% Glycerol, 5 mM imidazole, 2 mM β-mercaptoethanol, 5 mM IAM, 1 mM PMSF and 1 mM NEM). Lysates were centrifuged for 10 min at 21,000 g. The supernatant was pooled and incubated with Ni-NTA beads for 1 h at 4℃ on a rotator. Ni-NTA beads were then washed with wash buffer A (50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% Glycerol, 10 mM imidazole, 2 mM β-mercaptoethanol, 10 mM IAM, 1 mM PMSF and 1 mM NEM). Protein were eluted by elution buffer (50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% Glycerol, 0.5 M imidazole, 2 mM β-mercaptoethanol, 5 mM IAM, 1 mM PMSF and 1 mM NEM). After that, incubating the eluate with preequilibrated immobilized streptavidin beads and then rotating 2 hours at 4℃. The streptavidin beads were washed with wash buffer B (50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% Glycerol, 2 mM β-mercaptoethanol, 5 mM IAM), and then washed with wash buffer C (50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl) to remove detergent. The protein bound on the streptavidin beads was eluted by boiling in 2 x SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue, 1%-mercaptoethanol) 5 min at 80 ℃. Affinity

purified protein and its interactors were subjected to western blot analysis or LC-MS/MS analysis.

Western blot analysis

Proteins were separated on a 10% SDS-PAGE, and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and then probed with monoclonal antibody against streptavidin which HPR-conjugated (Abcam, Cambridge, UK) with 1: 1000 dilution. The signal was detected by chemiluminescence.

RNA isolation and qRT-PCR analysis

Yeast cells were grown to early log phase (OD_{600}=1.0), and then treated the cell 4 hours with 32 μg/mL fluconazole. Collected cells in these two stages and quickly frozen in liquid nitrogen. Briefly, grinded the cells in liquid nitrogen circumstance, lysed cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), denatured and removed protein with chloroform and then centrifugation at 12, 000g for 15 min at 4℃. Took aqueous phase mixed with precooled isopropanol to precipitate nucleic acids and centrifugation at 12, 000g for 10 min at 4℃. Discard the supernatant, and washed the sediment with precooled 75% ethanol before span at 7,500g for 8 min. Discarded all supernatant and dried at room temperature. Resuspended the nucleic acids with diethyl pyrocarbonate (DEPC) treated water. Digested DNA with RNase-free DNase I (Promega, Madison, WI, USA) at 37℃ for 30min. Repeated the above-mentioned steps to remove DNase I and gained pure RNA. RNA was resuspended in DEPC water. Mix 200ng isolated RNA with reverse transcriptional system in Revertra Ace qPCR RT Kit (TOYOBO, Tokyo, Japan) 37℃ for 15 min to reverse transcribe to yield complementary DNA (cDNA).

cDNA with SYBR Green Master Mix (TOYOBO, Tokyo, Japan) was loaded in triplicate onto a 96-well plate. Level of mRNA of each gene was determined by quantitative real-time PCR amplification and analyzed by the Stratagene Max-Pro (Mx3000P) software (version 4.0). The 18S rDNA was used as internal standard. The primer sequences used in this study are shown in Table S2.

Protein turnover profiling for monitoring the proteome dynamic degradation

We took two methods to analysis the dynamic degradation process of the proteome we interested (8,9).

Strategy one: Firstly, all strains we interested were cultured in SC heavy medium for approximately 9 generations to complete full prelabeling. Then, the labeled cells were transferred into new fresh SC light medium at OD_{600}=0.6, after three times wash at room temperature with SC medium without all amino acids. Yeast cells are harvested at 0, 15, 30, 60 and 120 min. The yeast cells were lysed by 8 M urea and 50 mM NH_4HCO_3. Took supernatant into new tubes and reduced with 10 mM dithiothreitol (DTT) for 30 min at 45 ℃ and subsequently carbamidomethylated by 20 mM IAM for 30 min at darkness. Diluted the concentration of urea to 4 M with 50 mM NH_4HCO_3, and digested protein with Lys-C (Meizhiyuan, Beijing, China) for 4-6 h at 37 ℃(10), the samples were further digested by trypsin (Meizhiyuan, Beijing, China) overnight at 37 ℃ after diluting the urea to 1M with 50 mM NH_4HCO_3(11). Add 1% formic acid to stop digestion. The peptides were desalted with C18 Sep-Pak SPE cartridge (Waters, Milford, MA, USA) and vacuum evaporated. The resulting peptides were analyzed by LC-MS/MS.

Strategy two: yeast cells were cultured in usual SC light medium until the OD_{600}=0.8 and treated with cycloheximide (CHX) to inhibit protein synthesis. Equal amounts of cells were harvested at 0, 30, 60, 120 and 240 min. The yeast cells were lysed by 8 M urea and 50 mM NH_4HCO_3. The protein concentration was quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, San Jose, CA, USA). Same amount of protein was applied for western blot analysis or LC-MS/MS analysis as mentioned above.

Mass Spectrometric Lipid Analysis

Quantification of sterol species was performed as described previously (12,13). Yeast cell pellets were resuspended in 100 µL 150 mM NH_4HCO_3 (pH 8.0) and disrupted by shaking with 0.5 mm glass beads. Took 70 µL supernatant into 3 mL extracting solvent ($CHCl_3$: CH_3OH=17:1), vortex 1 min to mix uniformity. Stand 120 min at room temperature for full extraction. Centrifuge 10 min at 3000 rpm, 4 ℃. After stratification, the chloroform phase was transferred to new glass tubes. After drying, the samples were store at -80 ℃ until LC-MS/MS analysis.

Sterol was dissolved in 7.5 mM ammonium acetate in chloroform/methanol/ 2-propanol (1:2:4, V/V/V) and analyzed by MRM in positive ion mode using the transitions $[ergosterol+H]^+$ at m/z=379.34 and $[ergosterol+NH4]^+$ at m/z=444.30 using a QTRAP® 4500 mass spectrometer (AB Sciex, MA, USA). Samples were loaded through a LC system (I-class Acquity ultra performance liquid chromatography, Waters, Milford, MA, USA) with an auto sampler. The mobile phase A was isopropanol/acetonitrile/formic acid (90:10:0.1, v/v/v) containing 10 mM ammonium formate. The mobile phase B was acetonitrile/water/formic acid (60:40:0.1, v/v/v) containing 10 mM ammonium formate. A BEH-C18 column (1.7 µm, 2.1mmID x 50 mm, Waters, Milford, MA, USA) was used for separation of lipids. The column was maintained at 55 °C. The UPLC separations were 20 min/sample using the following scheme: (1) 0 min, 70% B; (2) 2 min, 57% B; (3) 2.1 min, 50% B; (4) 12 min, 46% B; (5) 12.1 min, 30% B; (6) 18 min, 1% B; (7) 18.1 min, 70% B; (8) 20 min, 70% B. All the changes are linear, and the flow rate was set to 400 µL/min. Lipid species were identified and quantified through using MSFileReader (Thermo Fisher Scientific, San Jose, CA, USA), ALEX, and Orange software 2.6.

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Supplementary figures and legends

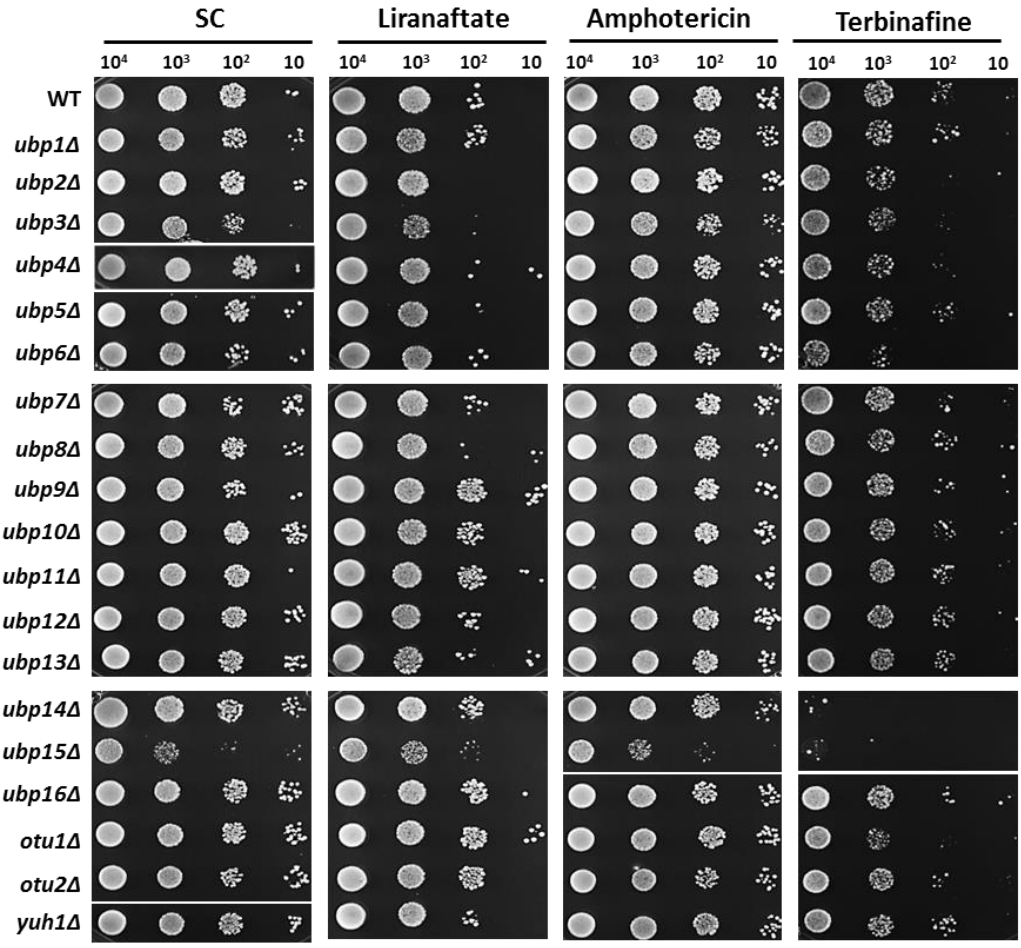

Figure S1. Phenotype assay of yeast DUB deletion strains with antifungal agents. Ten-fold dilution series of yeast WT and 19 DUB deletion strains were plated on SC plates (1st line), SC with 16 μg/mL Liranaftate (2nd line), 0.5 μg/mL Amphotericin (3rd line) or 128 μg/mL terbinafine (4th line). The plates were incubated at 30 ℃ for 72 hours. On the SC medium, WT to *ubp6Δ* including *ubp4Δ* was on the same plate; *ubp14Δ* to *yuh1Δ* was on the same plate. On the SC medium containing Amphotericin or Terbinafine, *ubp14Δ* to *yuh1Δ* was on the same plate. There was contaminant dot or blank line, so we cut off the figure.

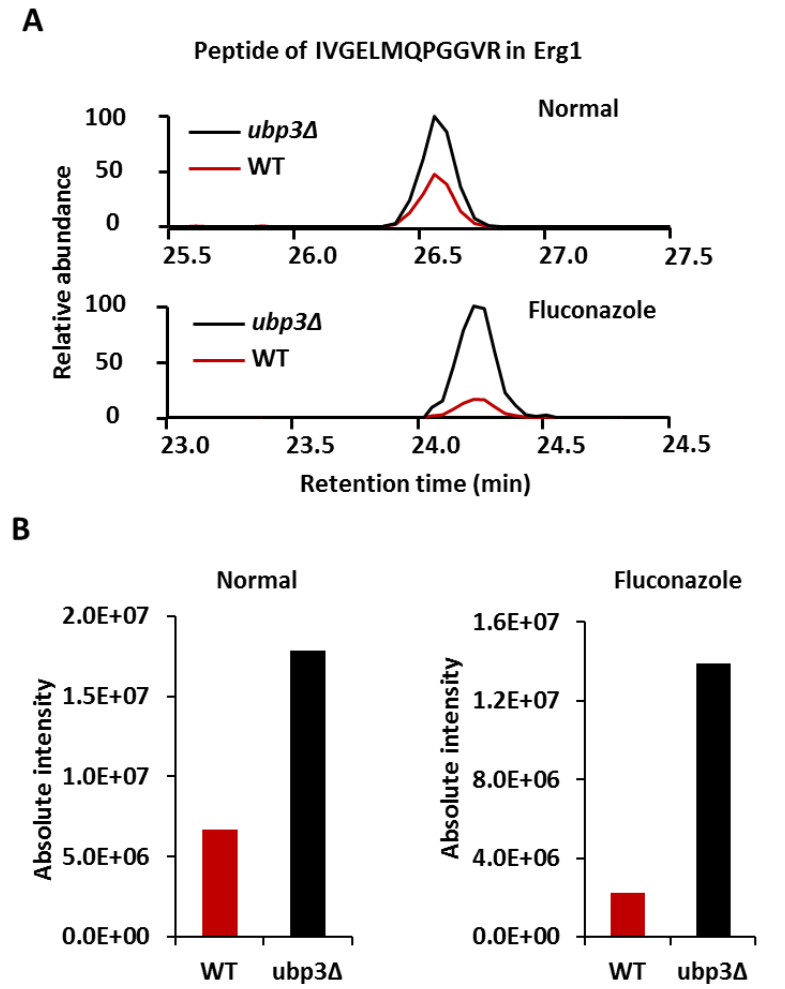

Figure S2. Loss of Ubp3 induced significant accumulation of Erg1 in normal or fluconazole condition. (A) The XIC of SILAC-paired unique peptide (IVGELMQPGGVR, charge = 2) from Erg1 in WT (red line) and *ubp3Δ* (black line) before and after fluconazole treatment. (B) The absolute intensity comparison of the unique peptide before and after fluconazole treatment.

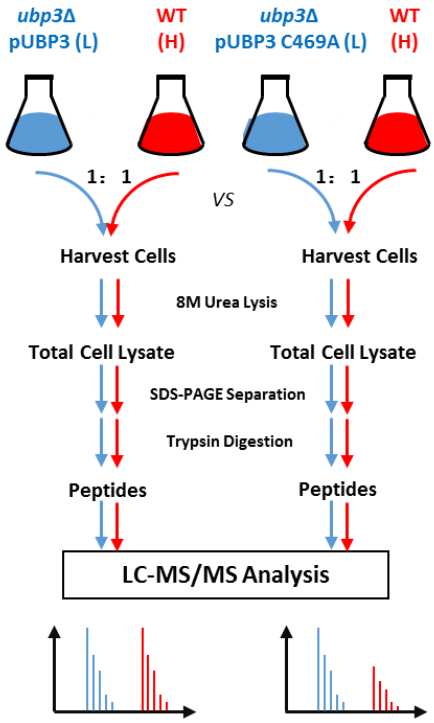

Figure S3. SILAC-based quantification of proteins in Ubp3 related strains and WT. Workflow to compare the protein level with Ubp3 replenished active or not. Label-swap experiments (forward and reverse) were made to increase the reliability of quality of the datasets. The *ubp3*Δ strain recovered UBP3 or catalytic active site mutant (Ubp3-C469A) was cultured and mixed with WT, respectively.

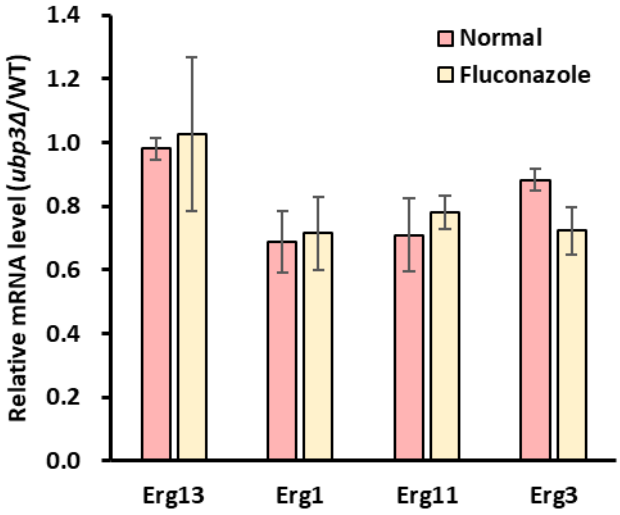

Figure S4. Relative mRNA ratios of key Erg enzymes in *ubp3*Δ and WT strains in normal and fluconazole treatment condition. Yeast cells were cultured to OD_{600}=0.8 and treated with 32 μg/ml fluconazole for 2 hours. Three biological replicates were performed.

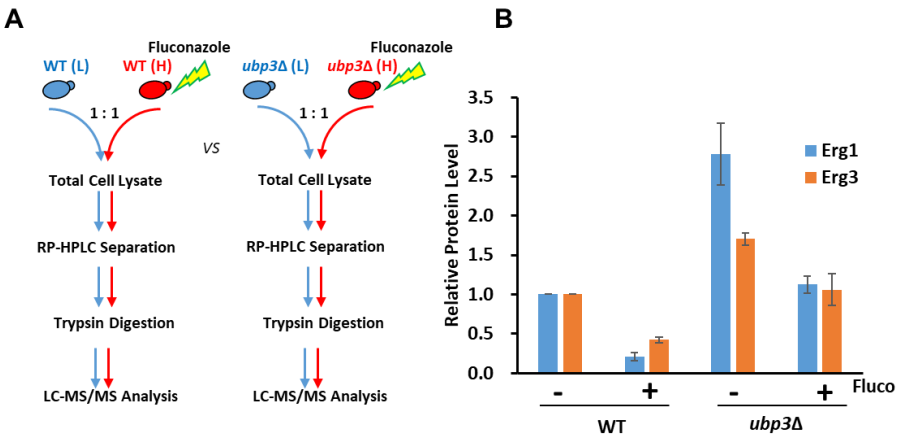

Figure S5. SILAC-based quantification to compare Erg proteins before and after fluconazole treatment. (A) SILAC-based quantitative proteomics to compare the proteome before and after fluconazole treatment in WT and *ubp*3Δ strains, respectively. Label-swapped experiments (forward and reverse) were performed. The yeast cell was treated with 32 μg/mL fluconazole for 4 hours. (B) Relative protein level of Erg1 and Erg3 before and after fluconazole treatment through SILAC-based quantification. The value represented the mean value of $\log_2$ ratio (*ubp3Δ*/WT) of quantified proteins in forward and reverse experiments. The $\log_2$ ratios were normalized on the value of WT without fluconazole treatment.

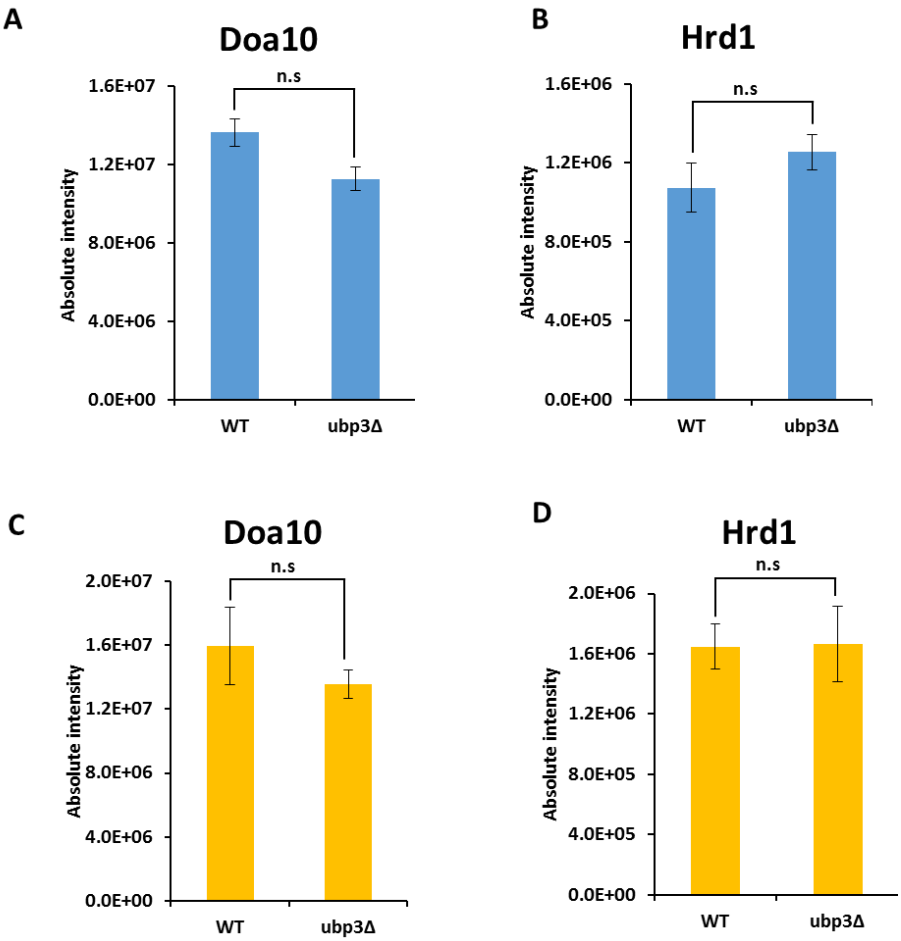

Figure S6. The abundance of ubiquitin ligases Doa10 and Hrd1 were not changed in Ubp3 absent or fluconazole treatment. (*A* and *B*) The abundance of Doa10 and Hrd1 in WT and *ubp3*Δ strains. The absolute intensity of Doa10 and Hrd1 were represented as the means (±SD) of forward and reverse experiment from figure 2A. (*C* and *D*) The abundance of Doa10 and Hrd1 in WT and *ubp3*Δ strains treated with fluconazole for 4 hours from figure 2*A*.

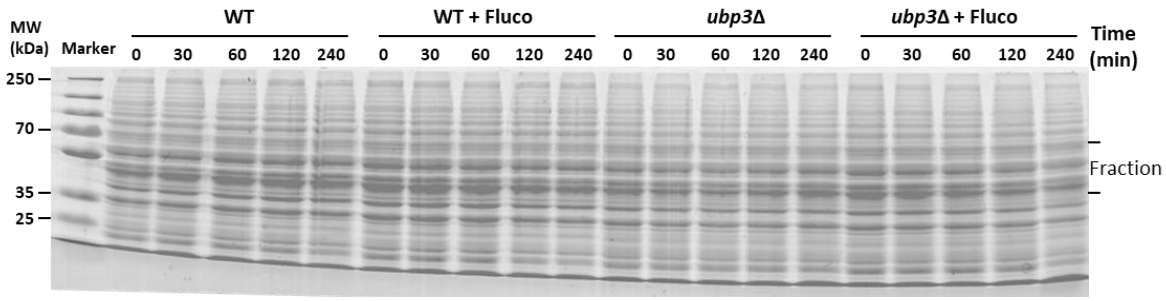

Figure S7. Dynamic degradation process of Erg enyzmes in the presence or absence of fluconazole. SDS-PAGE analysis of whole cell lysates of WT and *ubp3*Δ strains as indicated. The WT and *ubp3*Δ strains were cultured to OD_{600}=0.6 and treated with fluconazole with or without fluconazole for 2 hours. Then the cells were treated with cycloheximide (CHX) as the time presented. Whole-cell extracts were analyzed by LC-MS/MS and quantified through LFQ intensity.

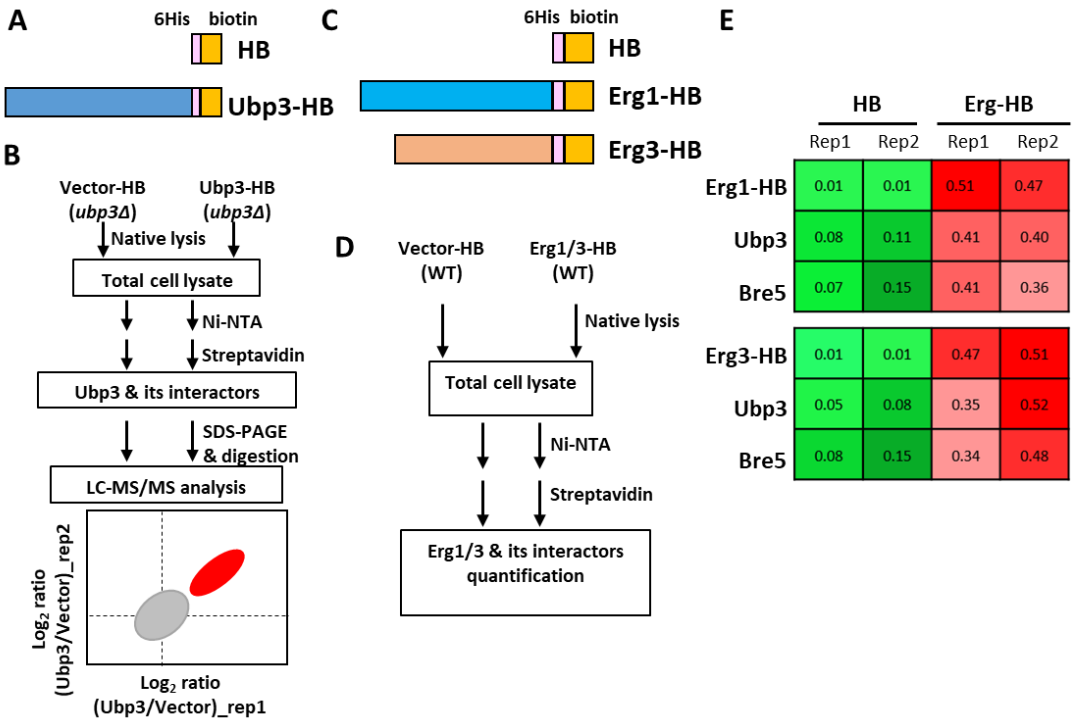

Figure S8. Ubp3 interacted with Erg1, Erg3 and proteasome. (A) Schematic for the construction of vector control (HB) and C-terminal His_6-biotin tagged Ubp3 (Ubp3-HB) expressed in *ubp*3Δ strain, respectively. (B) Interactome profiling of Ubp3 through label-free quantification. The His_6-biotin tagged Ubp3 (Ubp3-HB) on C-terminal and vector control (HB) were expressed in the *ubp*3Δ strain, respectively. Equal amounts of cells were harvested and lysed in native condition for tandem affinity purification. Two replications were performed for analysis. (C) Schematic for the construction of vector control (HB) and C-terminal His_6-biotin tagged Erg1 (Erg1-HB) and Erg3 (Erg3-HB) expressed in yeast strains, respectively. (D) Workflow of interactome profiling of Erg1 and Erg3 through label-free quantification. Equal amounts of cells were harvested and lysed in native condition for tandem affinity purification. Two replications were performed for analysis. (E) Proportion quantification of the interactors with Erg1 and Erg3.

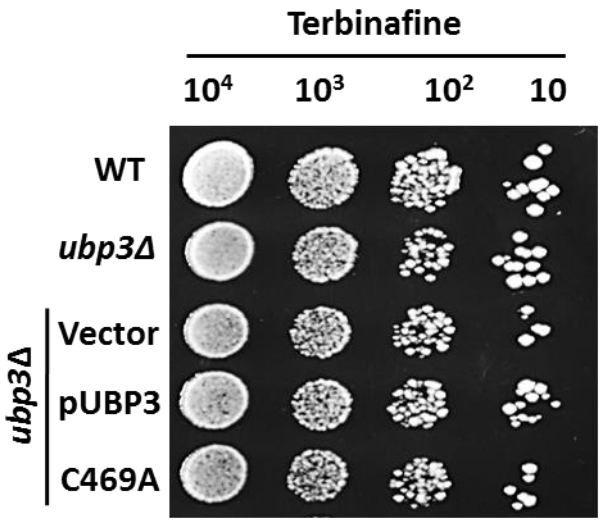

Figure S9. The phenotype of WT and Ubp3 related strains towards terbinafine.

Ten-fold serial dilutions of WT, *ubp3*Δ and its replenishing control (empty vector), UBP3 (pUBP3) and catalytic site mutant (C469A) strains were spotted on SC media containing 32 μg/mL terbinafine. The plates were incubated at 30 ℃ for 72 hours.