Ubiquitin Reference Technique and Its Use in Ubiquitin-Lacking Prokaryotes

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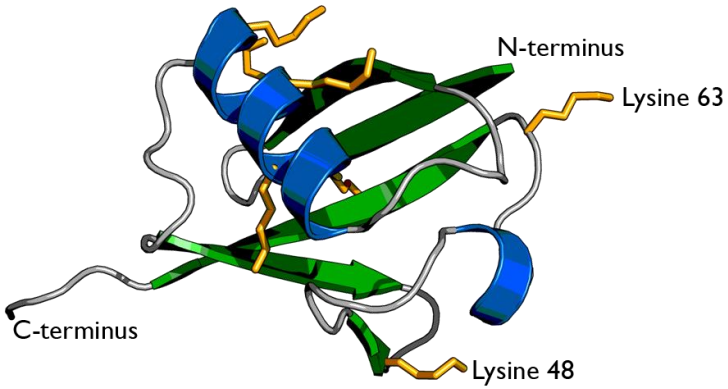
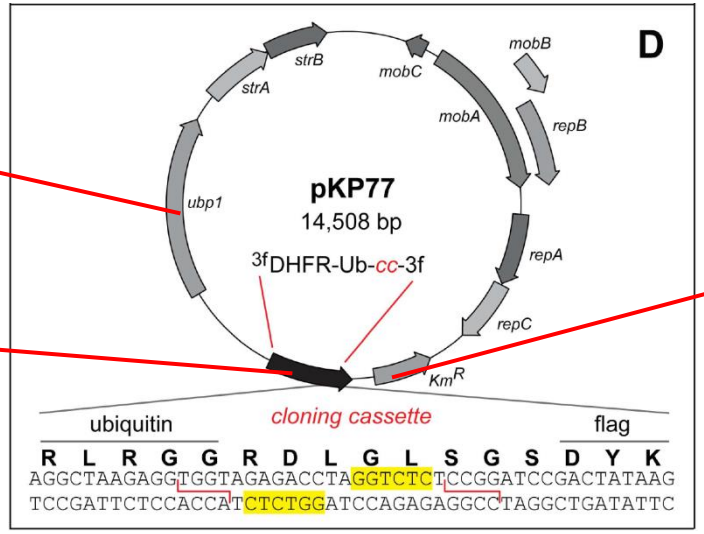


- Ubiquitin (76 aa)
- UB genes encode linear fusions of UB either to itself (poly-Ub genes) or to other proteins
- these fusions are cleaved by deubiquitylases (DUBs) yielding mature Ub

deubiquitylase

$FLAG_3$-DHFR-UB^{R48}



-X-βGal-$FLAG_3$

D. The broad host-range URT vector pKP77. It encodes the *S. cerevisiae* Ubp1 deubiquitylase (DUB) as well the ^{3f}DHFR-Ub^{R48} reference protein, followed by a DNA sequence containing a cloning cassette (*cc*) as well as a sequence encoding the triple flag tag. The cloning cassette contains two inverted BsaI sites (yellow rectangles). Digestion of pKP77 with BsaI generates unique cohesive ends (indicated by red lines) that allow precise, unidirectional insertion of a sequence of interest while preventing self-ligation of the cut plasmid (see Materials and Methods). Other notations on the map denote specific bacterial genes of the parental pJRD215 plasmid (GenBank accession number JX181778).

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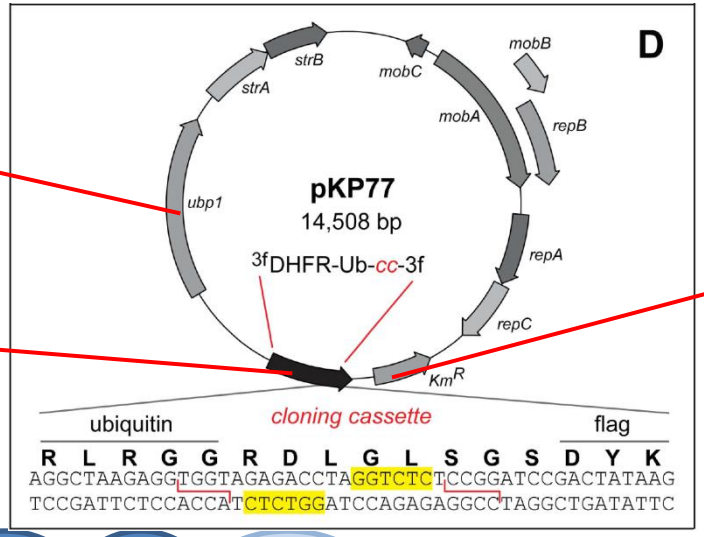
deubiquitylase

$FLAG_3$-DHFR-UB^{R48}



X-βGal-$FLAG_3$

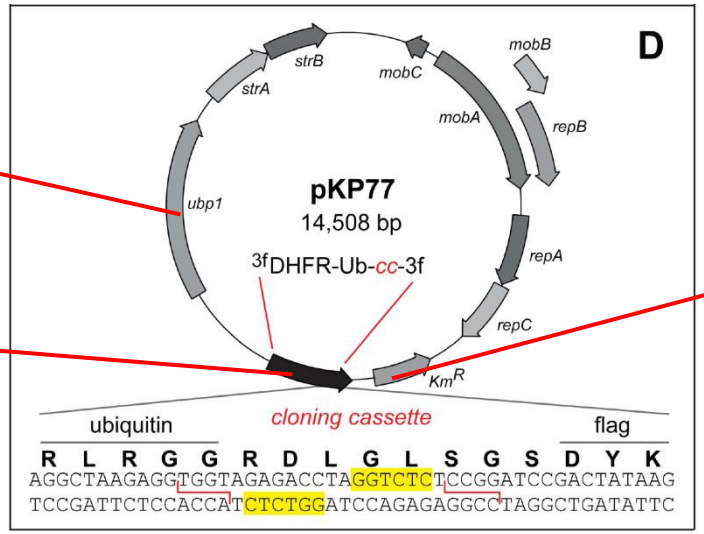
$FLAG_3$-DHFR-UB^{R48}X-βGal-$FLAG_3$

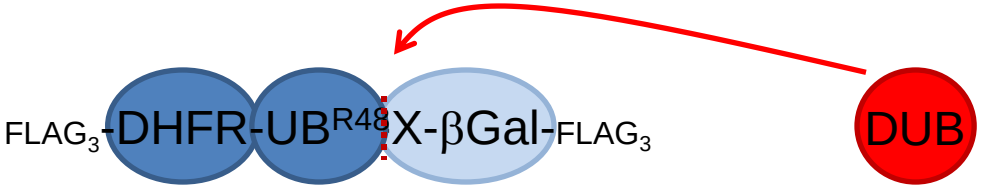
DUB

deubiquitylase

$FLAG_3$-DHFR-UB^{R48}

Protein-$FLAG_3$

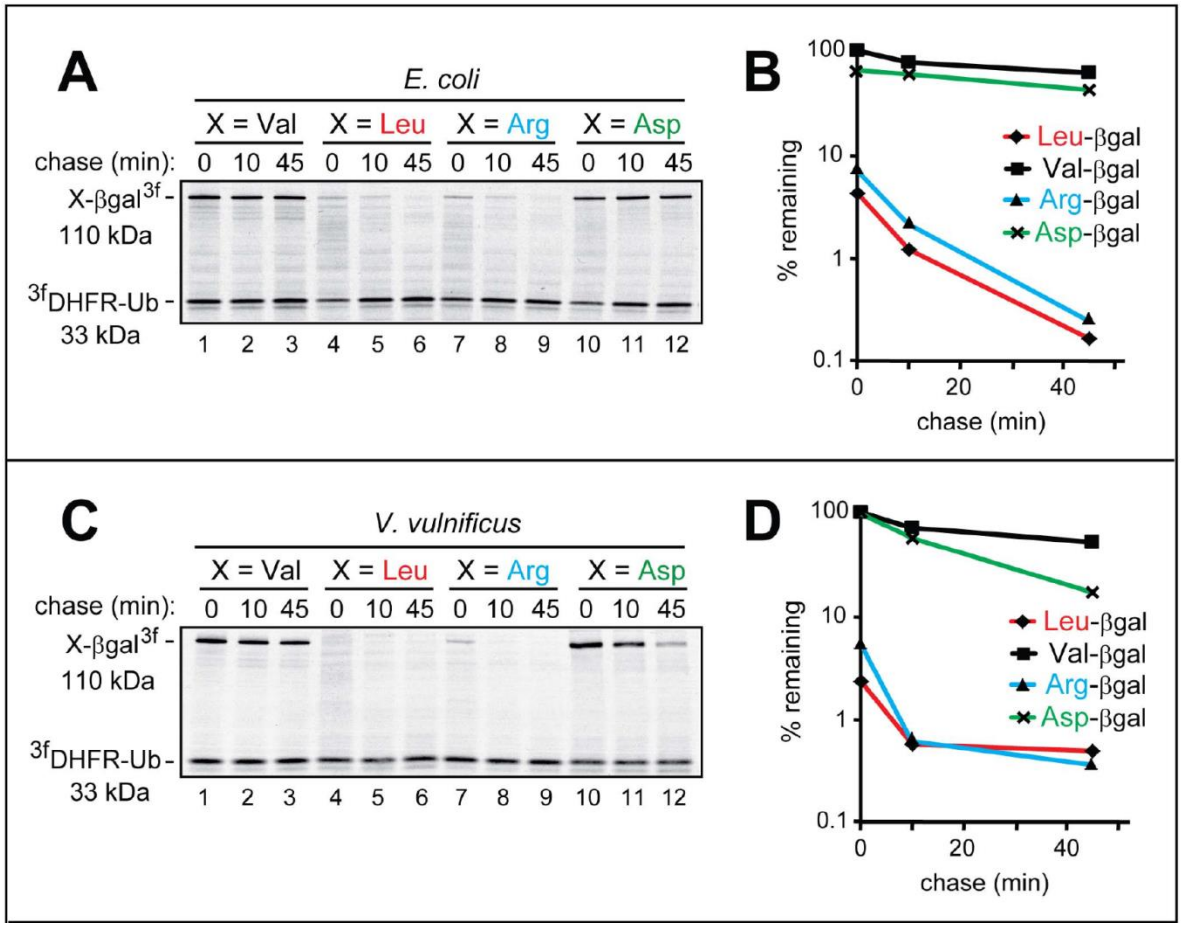




FLAG IP

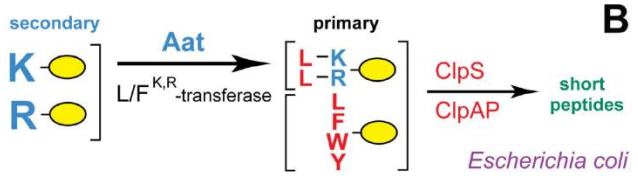
URT Pulse-Chase Assays and Immunoprecipitation

- *E. coli* and *V. vulnificus* were transfected with URT-based reporter plasmids
- cells were grown in LB at overnight,
- culture was diluted 1:200 in fresh LB and grown until A600 = 0.7
- 15 ml of cells were pelleted at 5000g for 5 min at RT, washed 3x with Pulse Medium (PM: M9 medium, pH 7.0, 0.5% glycerol, 0.5% glucose, 0.1 mM CaCl2, 2 mM MgSO4 and Methionine/Cysteine-free Synthetic Complete (SC) Mixture
- cells were resuspended in 135 µl of PM and incubated at 37°C for 10 min
- Proteins were pulse-labeled with 15 µl of Express [35S] Protein Labeling Mix (1.175 Ci/mmol, Perkin Elmer) for 3 min at 37°C
- labeling was quenched by the addition of 0.5 ml of Chase-Medium (CM: PM supplemented with Met and Cys at 0.5 mg/ml each) at 37°C
- at indicated times of chase cells were mixed with an equal volume of TDS buffer (1% SDS, 5 mM dithiothreitol (DTT), 50 mM Tris-HCl, pH 7.4, containing ‘‘complete protease-inhibitor mixture’’ (Roche)), followed by immediate freezing in liquid N2
- Frozen samples were directly heated at 95°C for 10 min, diluted with 10 volumes of TNN buffer (0.5% NP40, 0.25 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, containing ‘‘complete protease-inhibitor mixture’’ (Roche))
- lysate was added to 10 µl of magnetic beads linked to anti-flag antibody M2 (Sigma) and incubated at 4°C for 3 hrs
- IP was washed 4x in TNN buffer, resuspended in 20 µl of SDS-sample buffer, and incubated at 95°C for 5 min
- samples were fractionated by SDS-PAGE followed by autoradiography



The rule N-end pathway in E. coli

The Aat L/FR,K-transferase conjugates Leu to N-terminal Arg or Lys. Substrates bearing the indicated primary (bulky hydrophobic) destabilizing N-terminal residues are recognized by the ClpS N-recognin and are delivered to the ClpAP protease



The rule N-end pathway V. vulnificus

V. vulnificus, which contains both the Aat L/FR,K-transferase and the Bpt LD,E-transferase. As a result, N-terminal Asp and Glu, which are stabilizing(nondestabilizing) residues in E. coli, are secondary destabilizing residues in the V. vulnificus N-end rule pathway

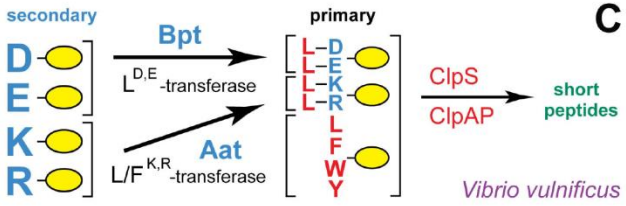


Figure 2. URT pulse-chase assays with model N-end rule substrates in *E. coli* and *V. vulnificus*. The set of URT-based ^{3f}DHFR-Ub^{R48}-X-βgal^{3f} fusions (X = Val, Leu, Arg, Asp) was assayed for the *in vivo* degradation of the released (by the yeast Ubp1 DUB) X-βgal proteins in *E. coli* (***A, B***) and in *V. vulnificus* (***C, D***) using ^{35}S-pulse-chases (***A, C***) and their quantification (***B, D***), as described in Materials and Methods. The bands of the 110 kDa X-βgal test proteins and the 33 kDa ^{3f}DHFR-Ub^{R48} reference protein are indicated on the left. Designations in ***B*** and ***D***: squares, Val-βgal; rhombs, Leu-βgal; triangles, Arg-βgal; crosses, Asp-βgal. ***E***. pKP55-X, encoding the *S. cerevisiae* Ubp1 DUB and ^{3f}DHFR-Ub^{R48}-X-βgal^{3f} URT-based fusions. Other notations on the map denote specific bacterial genes. The nucleotide sequences of pKP77 and pKP55-X are available in GenBank (JX181779 and JX181780). In addition, Table S3 contains the nucleotide sequence of pKP55-X.

Tandem fluorescent protein timers for *in vivo* analysis of protein dynamics

Anton Khmelinskii[1,2,7], Philipp J Keller[2,3,7], Anna Bartosik[1,2,8], Matthias Meurer[1,2,8], Joseph D Barry[4,8], Balca R Mardin[1], Andreas Kaufmann[2,6], Susanne Trautmann[2,5], Malte Wachsmuth[2], Gislene Pereira[5], Wolfgang Huber[4], Elmar Schiebel[1] & Michael Knop[1,2,5]

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analyze protein turnover and trafficking in vivo

- no pulse-chase metabolic labeling necessary (not possible in living cells)
- no photoactivation/photobleaching

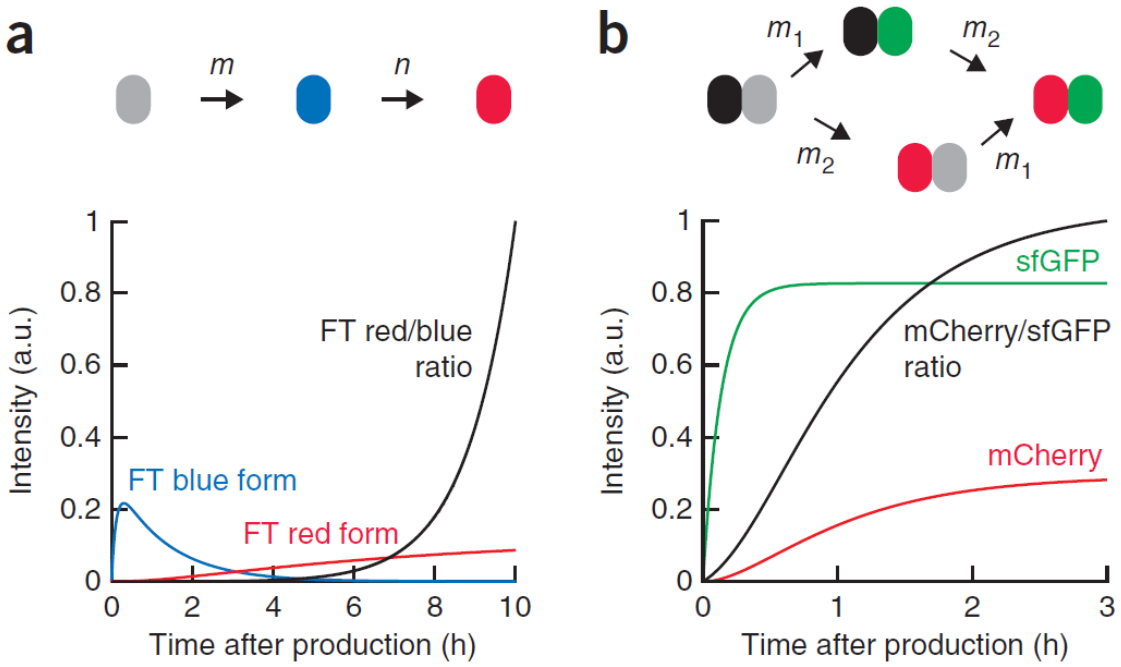
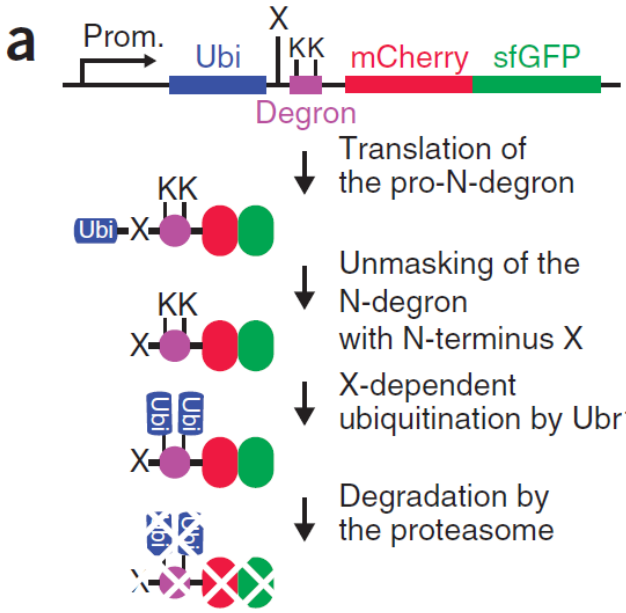
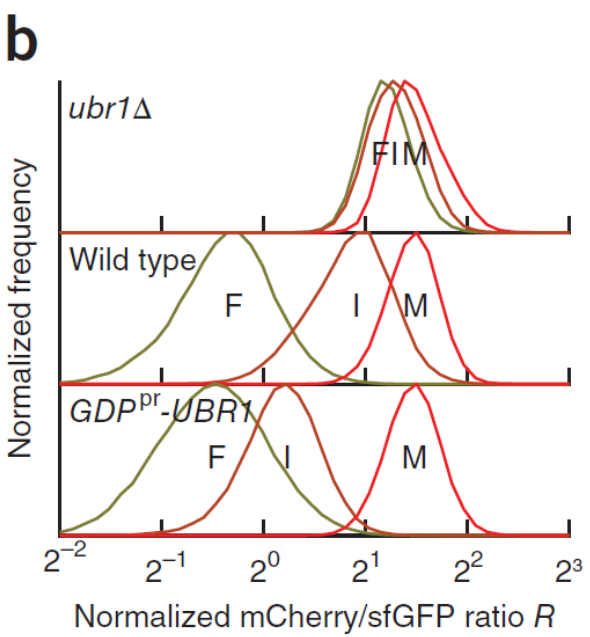


Figure 1 Tandem fluorescent protein fusions as fluorescent timers. (**a**) Illustration of a conventional fluorescent timer (FT) with maturation rate constants (*m*, *n*) of the fluorescent timer 'fast-FT'[15]. (**b**) Behavior of a tandem fusion of mCherry (black, red) and sfGFP (gray, green) with the maturation rate constants (m_1, m_2) determined in this study (**Supplementary Methods**). For simplicity, a one-step process represents mCherry maturation. Fluorescence intensity curves are normalized to the brightness of sfGFP. Ratios are normalized to the maximum in each plot.



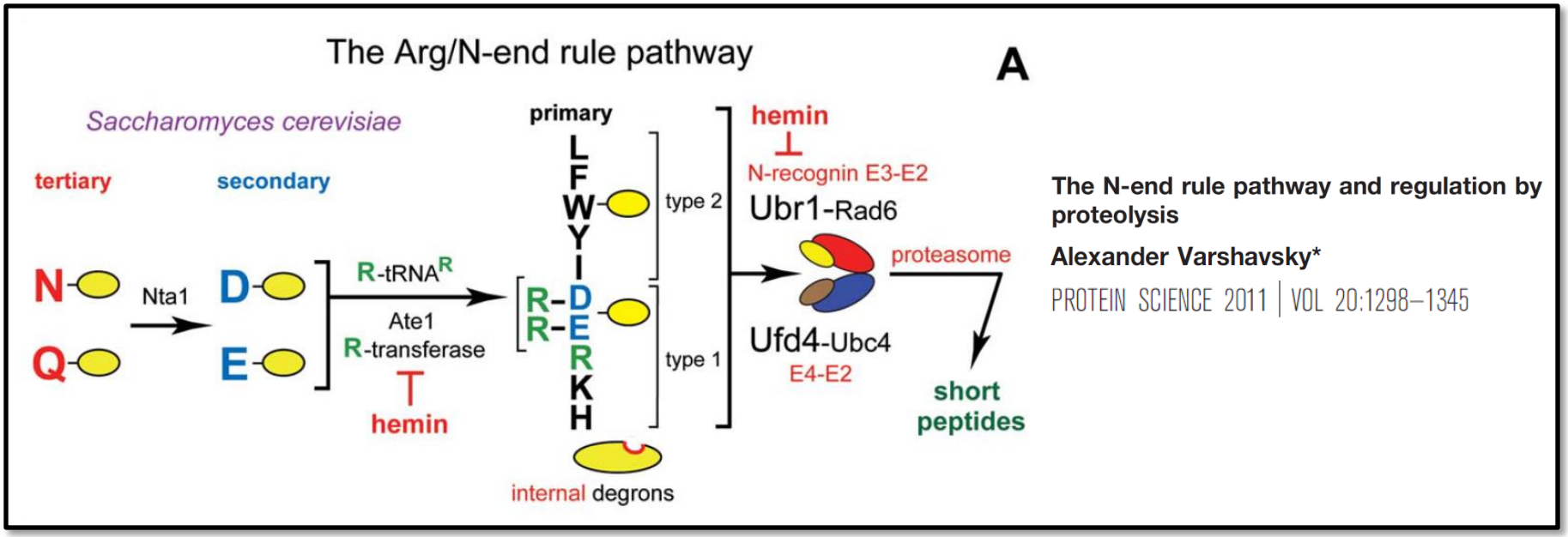


UBR1
E3 ubiquitin-protein ligase

← knockout

← wt

← overexpression



The N-end rule pathway and regulation by proteolysis

Alexander Varshavsky*

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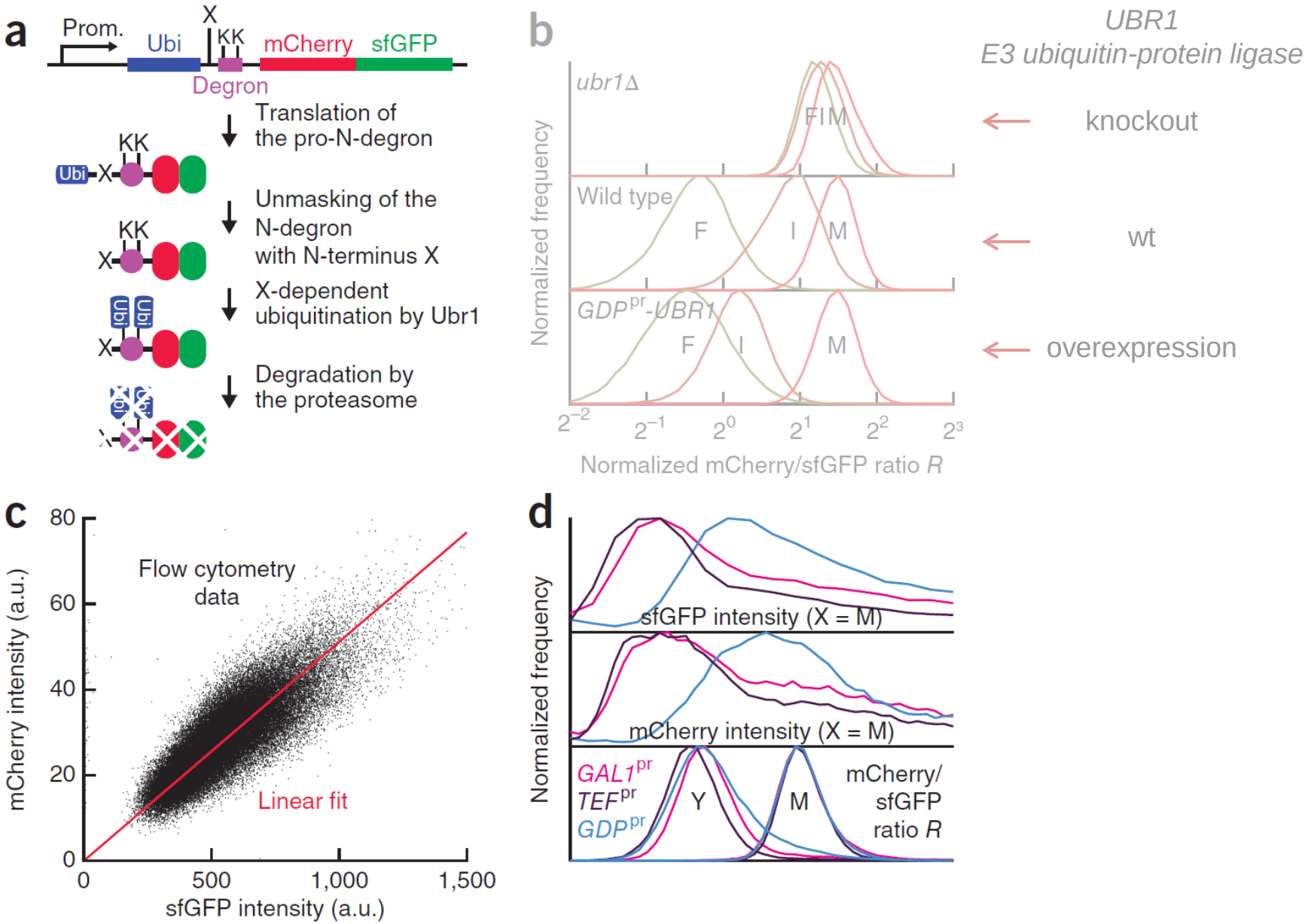

a
Prom.
Ubi
X
KK
mCherry
sfGFP
Degron
Translation of the pro-N-degron
Unmasking of the N-degron with N-terminus X
X-dependent ubiquitination by Ubr1
Degradation by the proteasome
b
ubr1Δ
Wild type
GDP^pr-UBR1
F
I
M
Normalized frequency
Normalized mCherry/sfGFP ratio R
UBR1
E3 ubiquitin-protein ligase
knockout
wt
overexpression
c
Flow cytometry data
Linear fit
mCherry intensity (a.u.)
sfGFP intensity (a.u.)
d
sfGFP intensity (X = M)
mCherry intensity (X = M)
GAL1^pr
TEF^pr
GDP^pr
Y
M
mCherry/ sfGFP ratio R
Normalized frequency

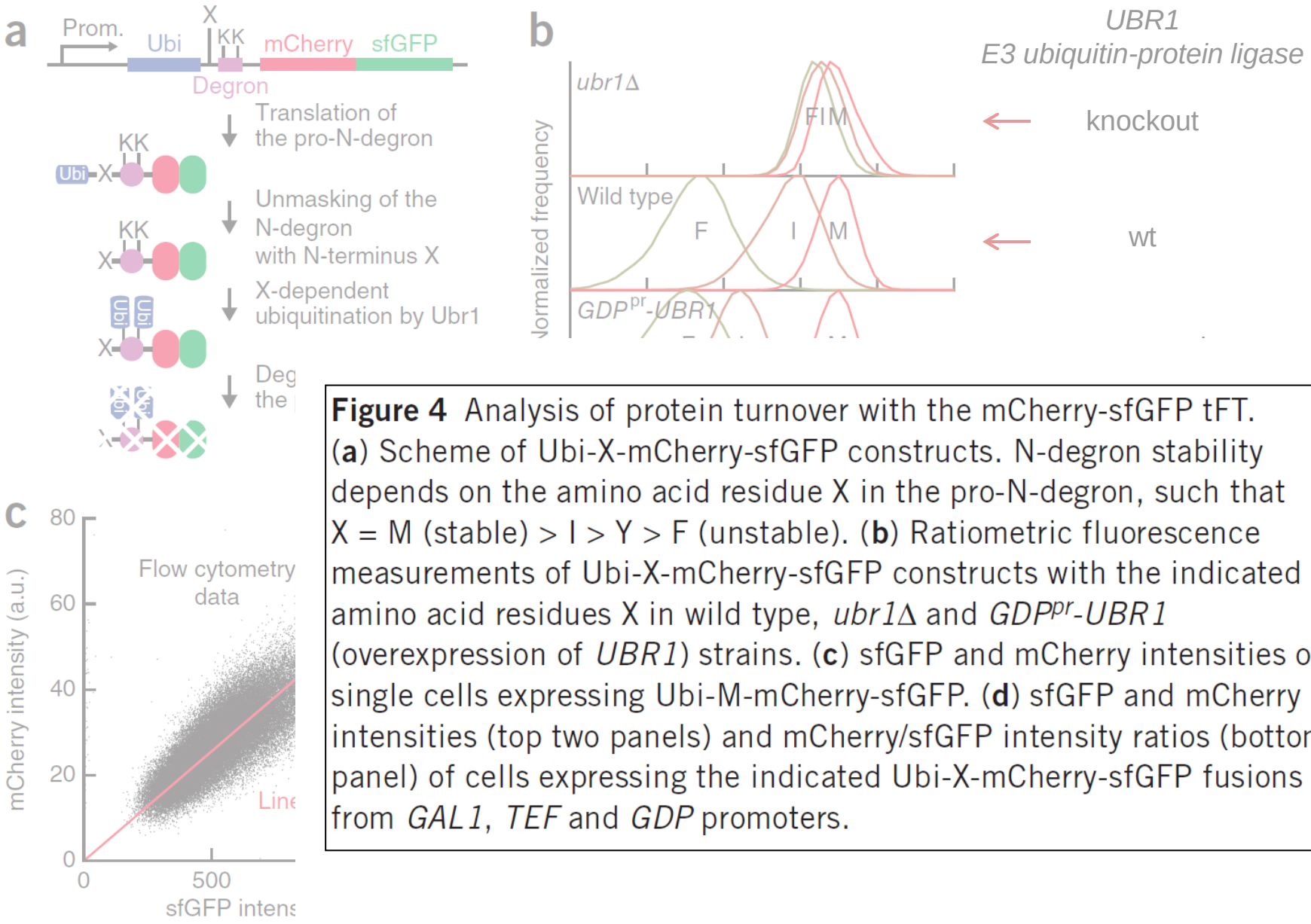
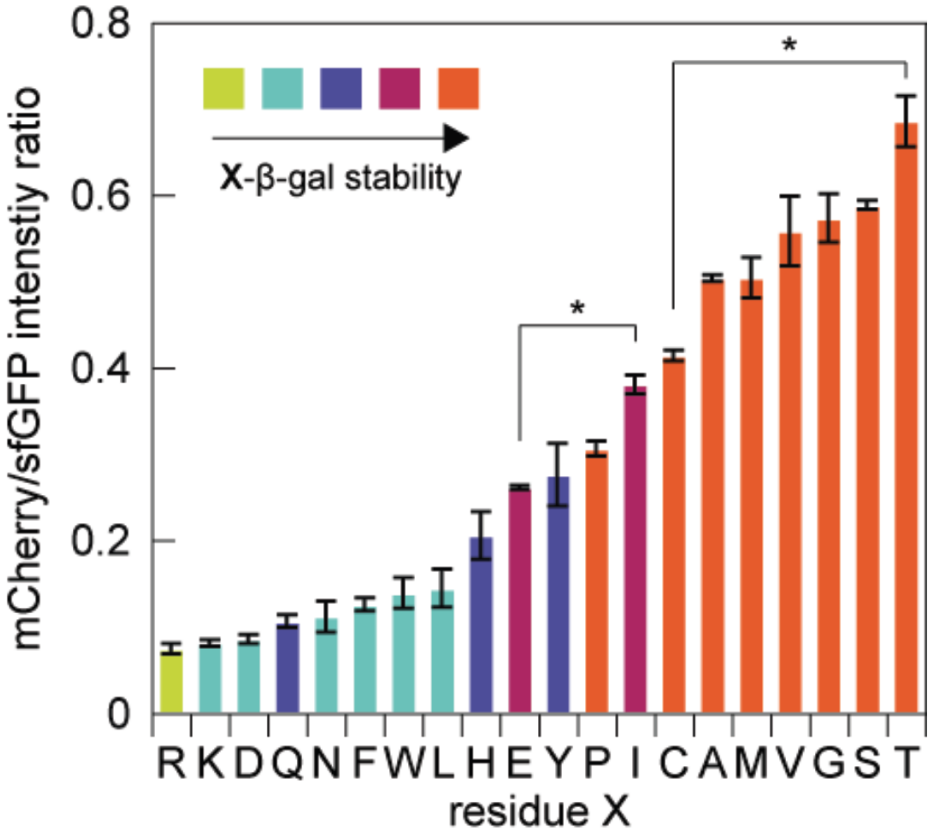


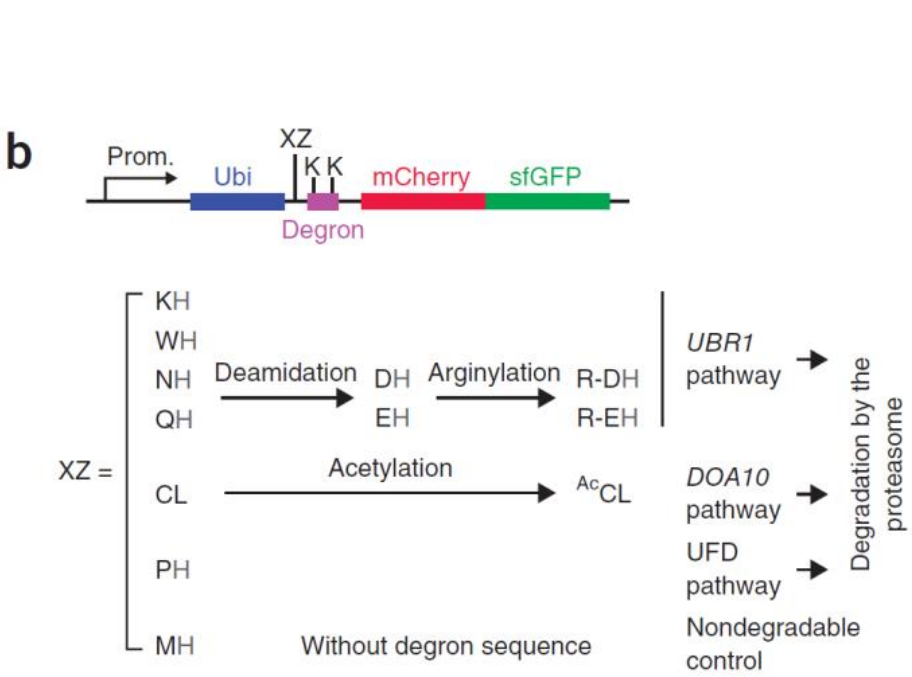
Figure 4 Analysis of protein turnover with the mCherry-sfGFP tFT. (**a**) Scheme of Ubi-X-mCherry-sfGFP constructs. N-degron stability depends on the amino acid residue X in the pro-N-degron, such that X = M (stable) > I > Y > F (unstable). (**b**) Ratiometric fluorescence measurements of Ubi-X-mCherry-sfGFP constructs with the indicated amino acid residues X in wild type, *ubr1*Δ and *GDP*^{pr}*-UBR1* (overexpression of *UBR1*) strains. (**c**) sfGFP and mCherry intensities of single cells expressing Ubi-M-mCherry-sfGFP. (**d**) sfGFP and mCherry intensities (top two panels) and mCherry/sfGFP intensity ratios (bottom panel) of cells expressing the indicated Ubi-X-mCherry-sfGFP fusions from *GAL1*, *TEF* and *GDP* promoters.



Supplementary Figure 7 | Ratiometric flow cytometry analysis of cells expressing Ubi-**X**-mCherry-sfGFP constructs with the indicated residues at position **X**. Ubi-**X**-mCherry-sfGFP constructs are colored according to the half-lives of corresponding **X**-β-galactosidase fusions, determined using pulse-chase experiments with metabolic labeling[25,25,26]. Measurement of the degradation kinetics of **X**-β-gal fusions led to the definition of the N-end rule, that grouped the twenty standard amino acids into five stability groups[25,26]. Our fluorescence measurements with flow cytometry recapitulated the pulse-chase results and revealed additional differences in stability conferred by amino acids within the same stability group. For example, N-terminal glutamic acid (E) was more destabilizing than isoleucine (I), or threonine (T) was more stabilizing than cysteine (C) (*, $p < 0.01$). Error bars indicate s.d.

High-throughput screening for regulators of protein turnover

- constructs with mCherry-sfGFP fused to different degrons were introduced into the SGA (synthetic genetic array) query strain Y8205
- each resulting query strain was crossed in triplicate with a genome-wide library of diploid yeast strains with heterozygous gene deletions
- The library was sporulated and spores were mated with the query strains using a RoToR pinning robot
- subsequent library manipulations—selection of diploids, sporulation, selection of haploids—were done using standard SGA protocols
- resulting haploid strains each carrying an mCherry-sfGFP fusion and a specific gene deletion were grown in 1,536-colony format for 24–48 h
- plates were imaged with a wide field IS4000MM-Pro fluorescence imager equipped with a 4-megapixel CCD camera and filters for sfGFP and mCherry fluorescence imaging (replicate 1) or with a wide field Decon imaging station equipped with an LED-based illumination system, a Retiga4000DC camera and filters for sfGFP and mCherry imaging (replicates 2 and 3)
- Images of a uniformly fluorescent plate were acquired for flat field correction
- For comparison between different plates/screens, the corrected measurements were normalized by dividing by the per-plate median absolute deviation (MAD) of corrected measurements
- normalized B-scores of all constructs were strongly affected in ~250 strains lacking genes mostly related to mitochondrial functions. These strains were identified and omitted from the analysis
- A gene deletion was considered to stabilize if
  - sfGFP intensity was increased (Δ-score sfGFP > 0) (increased abundance)
  - mCherry/sfGFP intensity ratio increased (Δ-score mCherry/sfGFP ratio > 0) (increased stability)



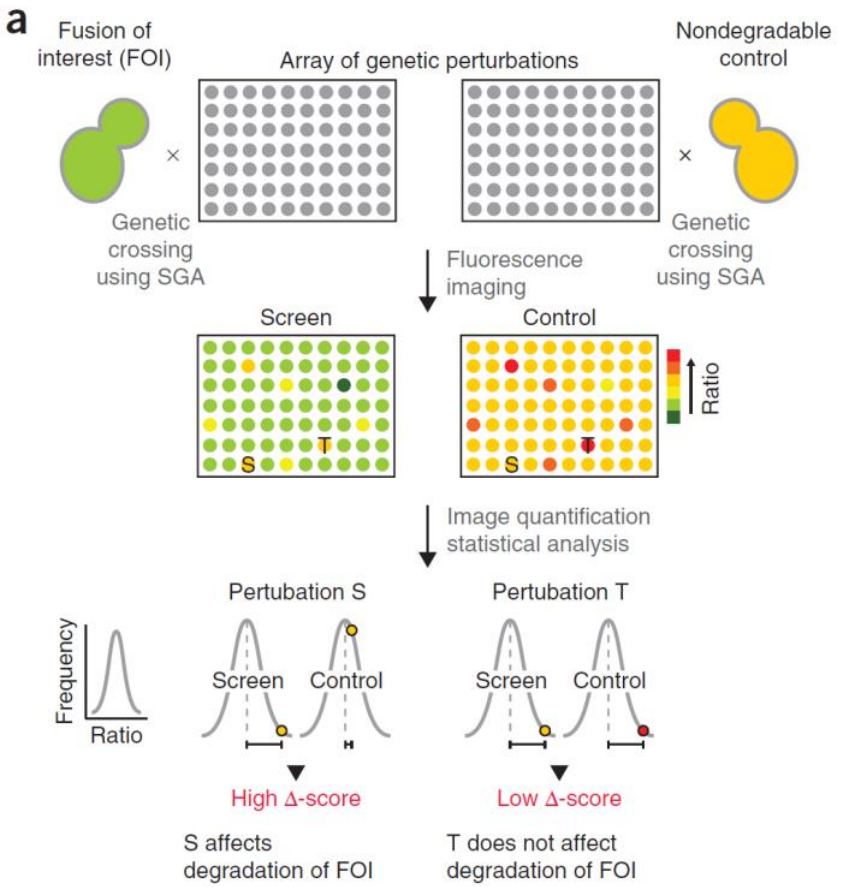
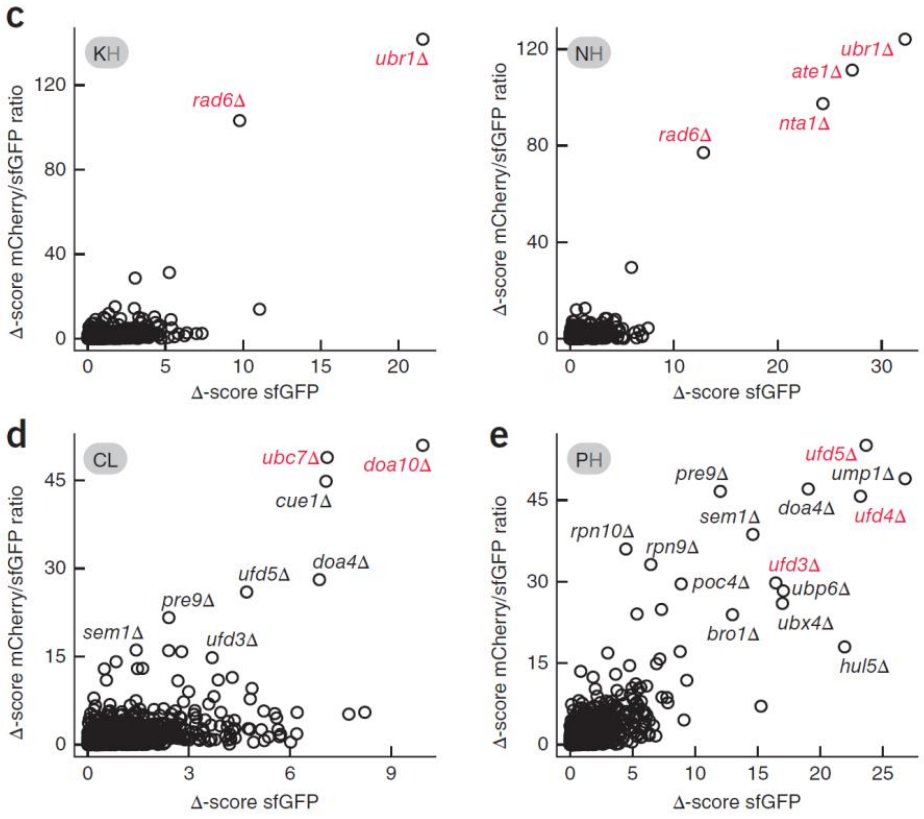
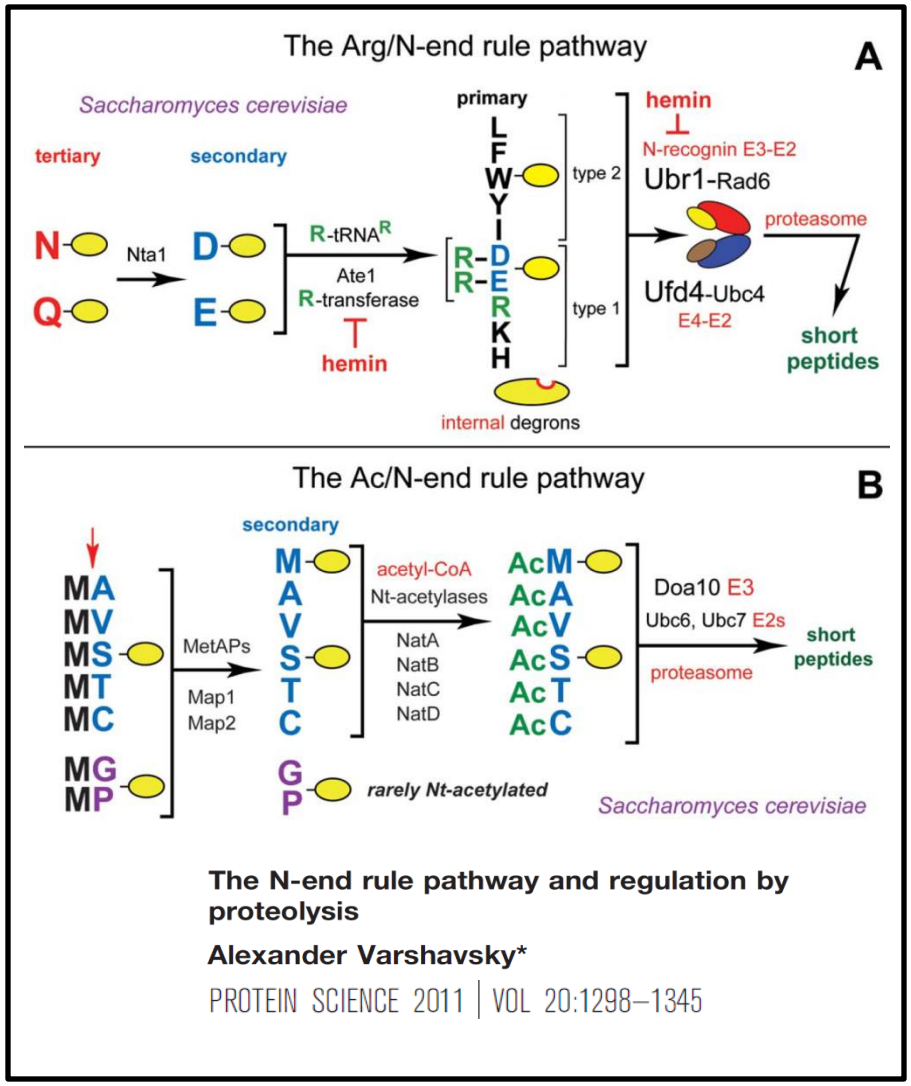


Figure 5 Identification of components of the N-end rule pathway. (**a**) High-throughput pipeline for systematic identification of regulators of protein turnover. The analysis of mCherry/sfGFP intensity ratios is exemplified for two genetic perturbations (S, T). (**b**) N-degrons used to screen for components of the N-end rule pathway. The two N-terminal amino acid residues define the N-degron recognized by Doa10 (residues XZ in the pro-N-degron). (**c**–**e**) Identification of gene deletions that stabilize the indicated N-degrons. Only gene deletions with positive Δ-scores are shown. Expected hits are highlighted in red. (**f**) Heat map of top gene deletions that increase the abundance and stability of each N-degron. The impact of each gene deletion is represented by a single color-coded value (*d*), which combines the Δ-scores of the sfGFP and mCherry/sfGFP channels (Online Methods). Gene deletion strains with $d \geq 6$ in at least one screen replicate were included in the heat map. Mutants of the ubiquitin-proteasome system (UPS) are indicated.





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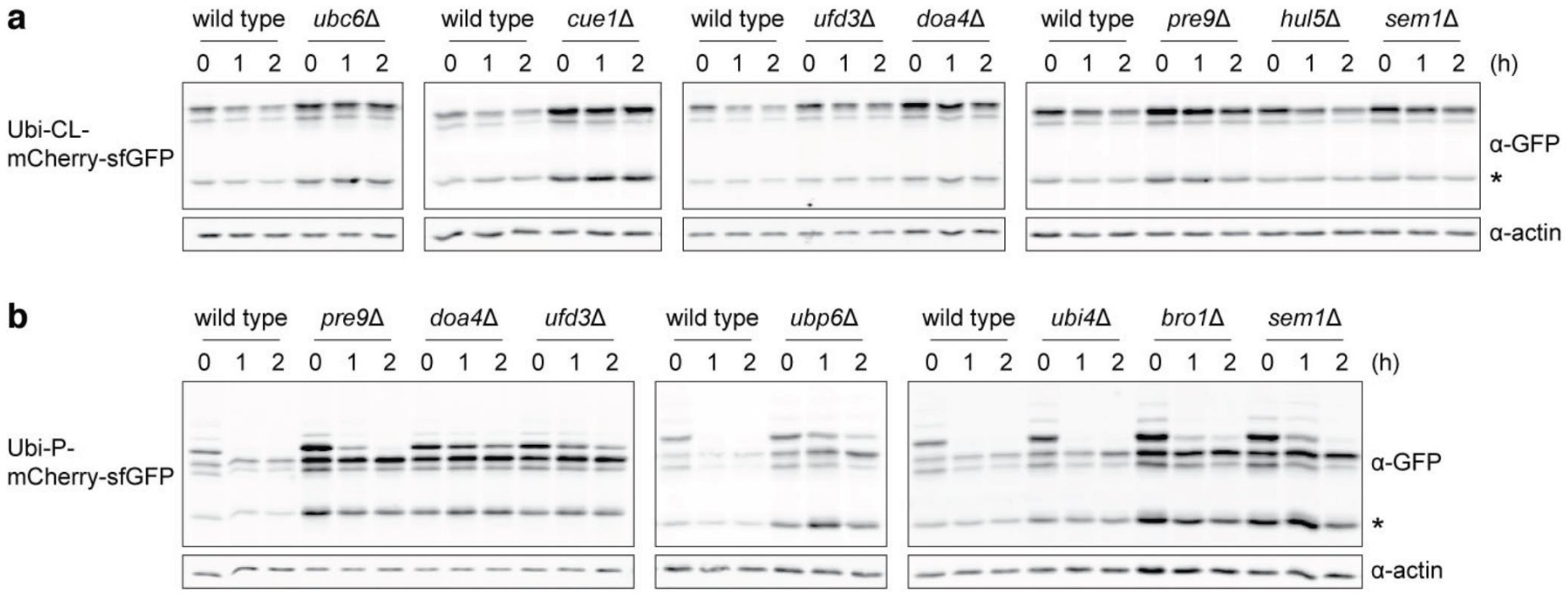

Supplementary Figure 14 | Validation of Ubi-CL-mCherry-sfGFP and Ubi-P-mCherry-sfGFP stabilization in strains lacking different components of the ubiquitin-proteasome system.
(**a**) Cycloheximide chase experiments with the indicated strains expressing Ubi-CL-mCherry-sfGFP. Strains were grown to OD_{600} 0.6-1.0 in SC-glucose medium at 30°C and cycloheximide (C7698, Sigma, Germany) was added to final concentration of 0.1 mg/ml. Samples were collected at the indicated time points by centrifugation at 3000 g for 5 min and flash frozen in liquid nitrogen. Whole cell extracts were prepared as previously described[18], separated by SDS-PAGE followed by semi-dry blotting and probed with rabbit anti-GFP and mouse anti-actin (mAB1501, Chemicon/Millipore) antibodies. Secondary antibodies labeled with $Alexa_{680}$ (Invitrogen, Germany) or $IRDye_{800}$ (Rockland Immunochemicals Inc., USA) were used for detection with an Odyssey Infrared Imaging System (LI-Cor Biosystems, Germany). Membranes were scanned at 700 and 800 nm wavelengths with a resolution of 169 µm in medium quality. A product of mCherry autohydrolysis during cell extract preparation[20] is marked with an asterisk (*). Ubi-CL-mCherry-sfGFP is visibly stabilized by deletion of *CUE1, UBC6, UFD3, DOA4, PRE9* and *SEM1*, consistent with the results from the screens (Fig. 5f).
(**b**) Cycloheximide chase experiments with the indicated strains expressing Ubi-P-mCherry-sfGFP analyzed as in (**a**). Ubi-P-mCherry-sfGFP is stabilized by deletion of *PRE9, DOA4, UFD3, UBP6, UBI4, BRO1 and SEM1*.