Supplemental Material for

Structural basis of substrate recognition and specificity in the N-end rule pathway

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Supplementary Figures 1–5
Supplemental Figure 1. Sequence and structural alignment of ubr boxes. (a) Sequence alignment of the ubr boxes of human UBR1 to UBR7 with identification of the zinc coordinating histidine and cysteine residues. Above the alignment, open boxes (α-helices) and arrows (β-strands) indicate the positions of regular secondary structure elements in UBR1. Vertical arrows highlight the residues involved in recognition of the N-degron N-terminus (red) and penultimate residue (black). (b) Structural alignment of UBR1 and UBR2. The ubr box of UBR2 protein is rainbow colored form blue (N-terminal) to orange (C-terminal) and the ubr box of the UBR1 is represented in white. Overall, a similar structural conformation was observed for both unbound ubr boxes with an RMSD of 0.58 Å for the superposition of Cα atoms.
Supplemental Figure 2. Structure of the UBR1 ubr box with intermolecular interactions that mimic N-degron binding. (a) Electrostatic potential surface representation of the UBR1 ubr box with N-terminal residues, Gly-Ser, from the adjacent molecule shown in green. (b) Electron density omit map (2Fo - Fc, 1σ contour) of the GS peptide bound to the ubr box. The key side chains involved in peptide binding are shown. (c) Ubr box recognition elements that hydrogen bond and stabilize the N-terminal and second peptide residues. Atoms are coded by color: oxygen (red), nitrogen (blue), carbon (green for peptide, white for UBR2). (d) Schematic of the elements responsible for N-degron recognition. Hydrogen bonds are represented by dashed straight lines and hydrophobic interactions represented by dashed curves.
Supplemental Figure 3. \(^{15}\text{N}-^{1}\text{H}\) correlation spectra of the ubr box from UBR2 titrated with carboxyl amidated amino acids. (a) Titration with Arg\(^{\text{NH2}}\) shows specific binding. (b) Plot of the magnitude of the largest amide NMR chemical shift changes as a function of Arg\(^{\text{NH2}}\) concentration. The binding affinity was determined from the curve of best fit taking into account the concentration of protein and added Arg\(^{\text{NH2}}\). (c) Titration with Lys\(^{\text{NH2}}\). (d) Titration with His\(^{\text{NH2}}\). Similar results were observed with the UBR1 ubr-box. Affinities are reported in Table 2.
**Supplemental Figure 4.** Isothermal titration calorimetry traces for the binding of different tetrapeptides to the UBR2 ubr box. The upper curves present the baseline corrected thermograms and the lower curves show the integrated areas of the heat released or absorbed (positive values and upward deflections) along with the best fits calculated using the manufacturer's software. Boxed values present the stoichiometry (N), molar association constant (K), enthalpy (ΔH), and entropy (ΔS) from the best fit.
Supplemental Figure 5. Mutagenesis confirms the role of key residues in N-degron-binding. (a) Loss of His136 leads to a loss of ubr box folding. (b) Expansion and overlay of the upfield $^1$H spectra of UBR1 ubr-box wild-type (WT) and two representative mutants upon titration with different N-end rule peptides. The spectrum in dark blue is unbound form. The spectrum in magenta is after addition of FIFS (a type 2 N-degron that does not bind significantly), green is after addition of RAFS (an intermediate high affinity ligand), and red is after addition of RIFS (a high affinity ligand).