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Supplemental Information

Architecture of the Ubiquitylation Module of the Yeast Ccr4-Not Complex

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SUPPLEMENTAL FIGURES AND LEGENDS

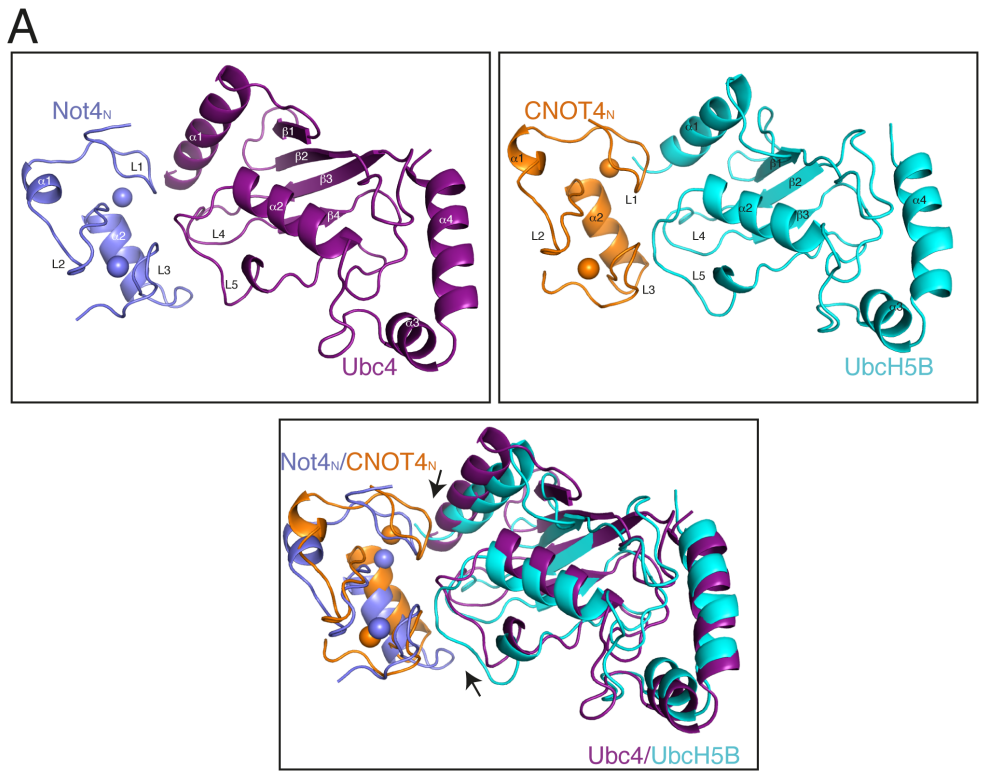

Figure S1

Detailed analysis of the Not4$_N$-Ubc4 crystal structure (Related to Figure 1)

(A) Not4$_N$-Ubc4 crystal structure and CNOT4-UbcH5B model are shown in similar orientation in the top panels. Superposition of the same is shown in the bottom panel. The difference in

the orientation of helix α1 of E2 and the loop regions of E3 at the interface are highlighted by arrows.

(B) Structure-based sequence alignment of all the E2 enzymes in *S. cerevisiae*. Not4 interacting residues of Ubc4 are indicated with blue dots. Residues providing specificity for this E2-E3 interaction are highlighted. The secondary structure elements are shown above the sequence.

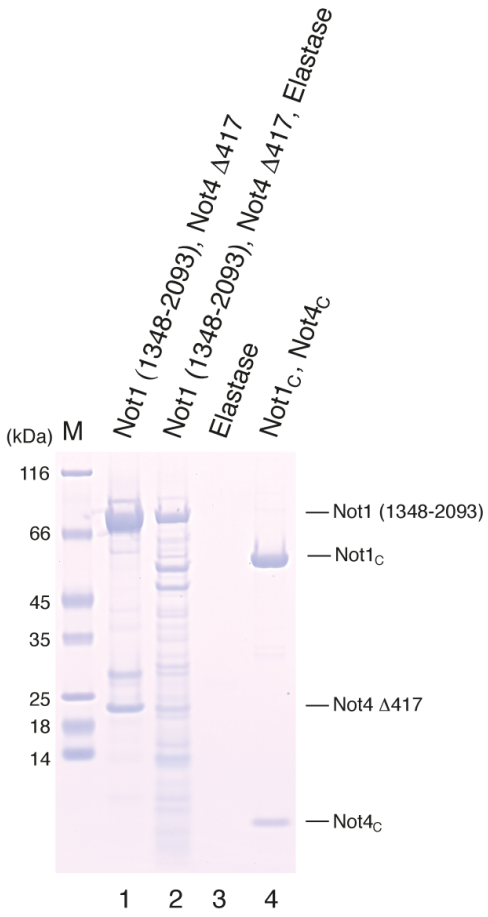

Figure S2

Identification of the $Not1_C$-$Not4_C$ minimal complex (Related to Figure 2)

Not1 (1348-2093)-Not4 Δ417 complex is shown in lane1. Limited proteolysis of Not1 (1348-2093)-Not4 Δ417 was carried out by incubating the complex at 0.6 mg ml^{-1} with elastase (Roche) for 60 minutes on ice at an enzyme to protein ratio of 1:10 and is shown in lane2. The mixture was then subjected to size-exclusion chromatography in a buffer containing 20 mM Tris-Cl pH 7.5, 250 mM NaCl and 2 mM DTT. The peaks were analyzed on 4-12% Bis-Tris NuPage gel with MES-SDS as the running buffer. The interacting fragments were identified by N-terminal sequencing and Liquid chromatography-Mass spectrometry (LC-MS) analysis. $Not1_C$-$Not4_C$ complex that was used for structural studies is shown in lane4.

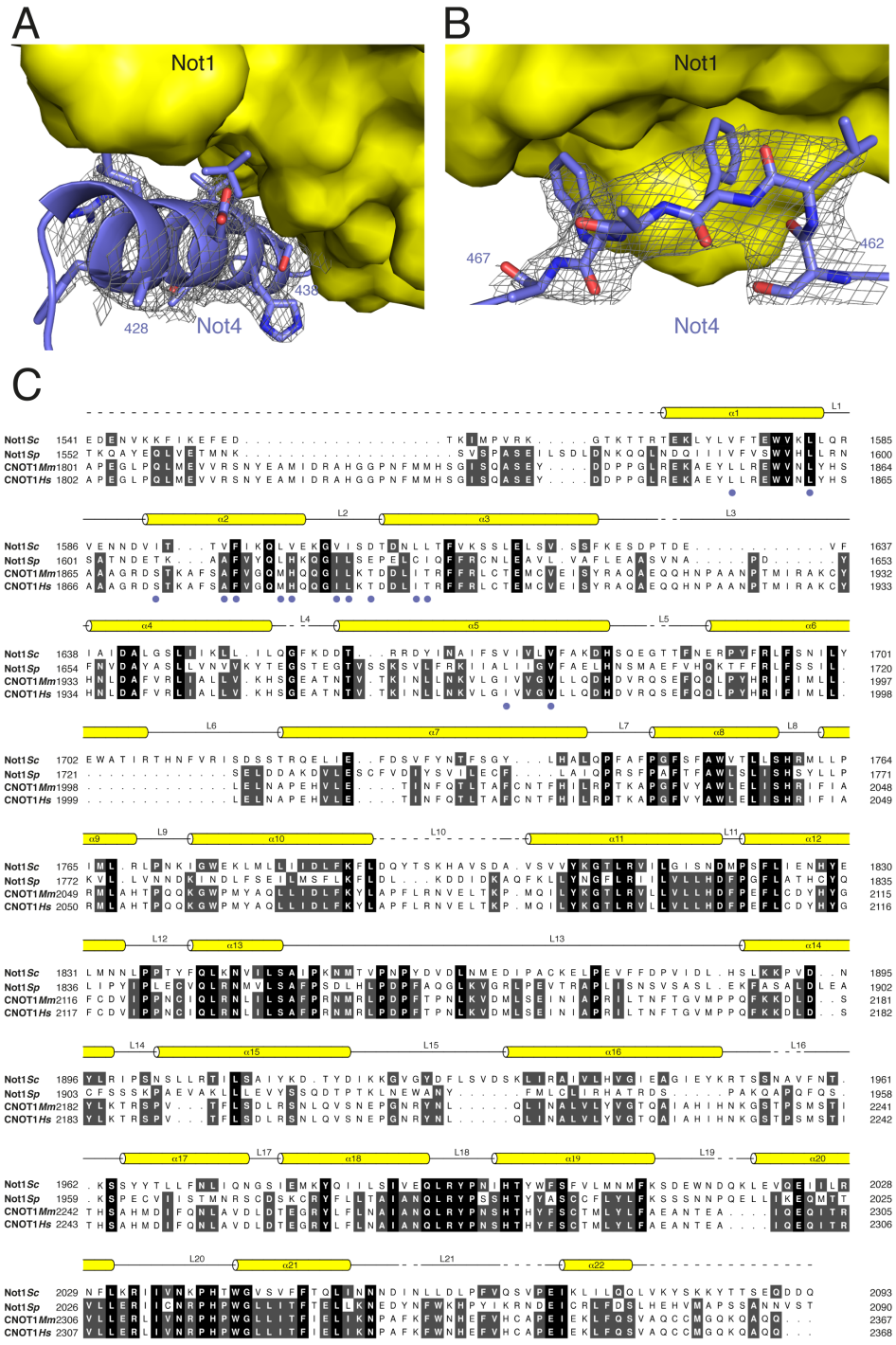

Figure S3

Detailed analysis of the Not1$_C$-Not4$_C$ crystal structure (Related to Figure 3)

(A-B) 2F_O-F_C electron density of Not4$_C$ at the hydrophobic interaction segments contoured at 0.9σ (corresponding to Figure 3A and 3C).

(C) Structure-based sequence alignment of Not1$_C$ from different species, including *S. cerevisiae* (*Sc*), *M. musculus* (*Mm*) and *H. sapiens* (*Hs*), highlighting the interacting residues with blue dots. The secondary structure elements are shown above the sequence.

SUPPLEMENTAL EXPERIMENTAL PROCEDURE

Protein purification

All proteins were cloned and expressed in *E. coli* BL21 pLysS cells (Stratagene) in TB medium with 0.5 mM IPTG induction overnight at 18 ˚C. Not1 constructs were expressed as previously described in (Bhaskar et al., 2013). $Not4_N$ and full-length Ubc4 were expressed as a fusion protein (connected by the linker TGSTGSTETG) with a N-terminal His-SUMO tag cleavable by Senp2 protease. The $Not4_C$, $Not4_C$–ΔN and $Not4_C$–ΔC (Not4 residues 418-477, 442-477 and 418-462, respectively) constructs were expressed as N-terminal His-GST fusion proteins followed by a 3C cleavage site. The proteins were purified using similar protocols as previously described (Bhaskar et al., 2013). Briefly, a first step of Nickel-based affinity chromatography was followed by tag cleavage and size-exclusion chromatography. For pull-down experiments, the GST-tagged proteins were purified with the same protocol but omitting the tag cleavage step.

Crystallization

The $Not4_N$-Ubc4 complex was crystallized at 48 mg ml^{-1} by vapour diffusion using 10% (w/v) PEG 8000, 0.02 M L-Na-Glutamate, 0.02 M Alanine (racemic), 0.02 M Glycine, 0.02 M Lysine HCl (racemic), 0.02 M Serine (racemic), 0.1 M Bicine/Tris-Cl pH 8.5 and 20% (w/v) ethylene glycol as crystallization buffer at room temperature.

$Not1_C$-$Not4_C$ complex was crystallized at 12 mg ml^{-1} by vapour diffusion using 10% (w/v) PEG 4000, 0.02 M 1,6-Hexanediol, 0.02 M 1-Butanol, 0.02 M 1,2-Propanediol (racemic), 0.02 M 2-Propanol, 0.02 M 1,4-Butanediol, 0.02 M 1,3-Propanediol, 0.1 M MOPS/Hepes-Na 7.5 and 20% Glycerol as crystallization buffer at room temperature.

Pull-down assays

100 pmol of GST-tagged protein was incubated with 200 pmol of the untagged prey protein for 1hr at 4 ˚C in the binding buffer (BB150 – 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 2 mM DTT, 12.5% (v/v) glycerol and 0.1% (w/v) NP40). 400 µL of BB150 buffer and 20 µL of 50% GSH-Sepharose resin were added to the protein mix and incubated for 1 hr with gentle rocking at 4 ˚C. The resin was washed 3 times with BB150 and the proteins were eluted with 15 µL of BB150 containing 20 mM Glutathione. Input and precipitate were mixed with 3X SDS loading dye and resolved on 4-12% Bis-Tris NuPage gel (Invitrogen) using MES-SDS as running buffer, and visualized by Coomassie blue staining.