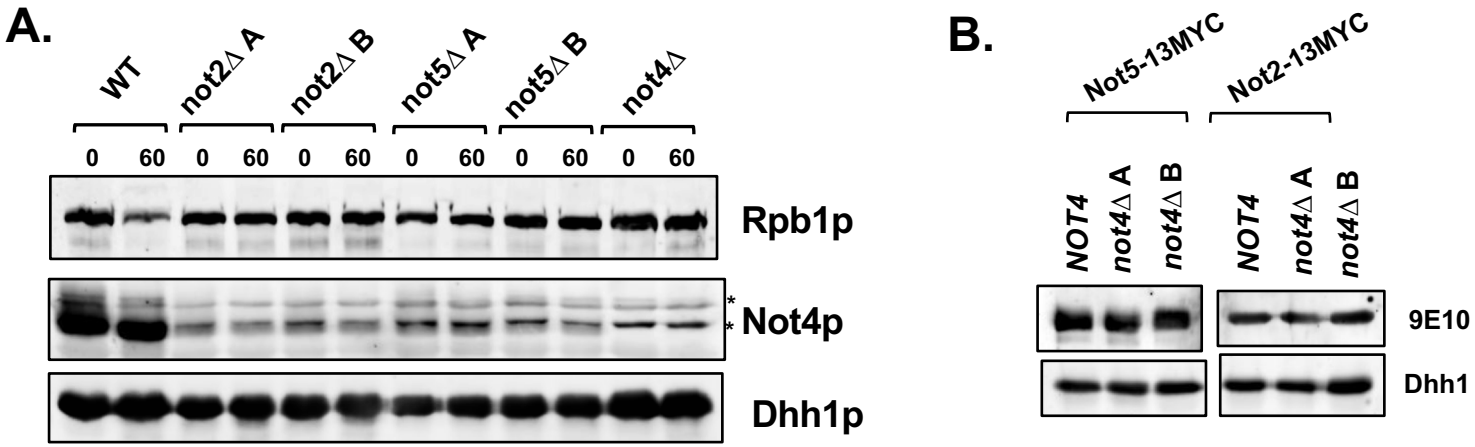


Figure S1. Deleting *NOT2* and *NOT5* reduces Not4p levels. (A). Western blot of wild type and *not2*Δ and *not5*Δ mutants. Two different isolates of each mutant are shown, labeled A and B. Cells were treated with 5ug/ml 4-NQO and 100ug/ml cycloheximide for 60 min. Rpb1 was detected using 8WG16. Dhh1 was used as the loading control. Asterisks indicate proteins cross-reacting with the Not4 antibody. (B). Deletion of NOT4 does not reduce Not2p or Not5p levels. Western blot for myc-tagged Not2 and Not5. Two different isolates of each mutant are shown, labeled A and B.

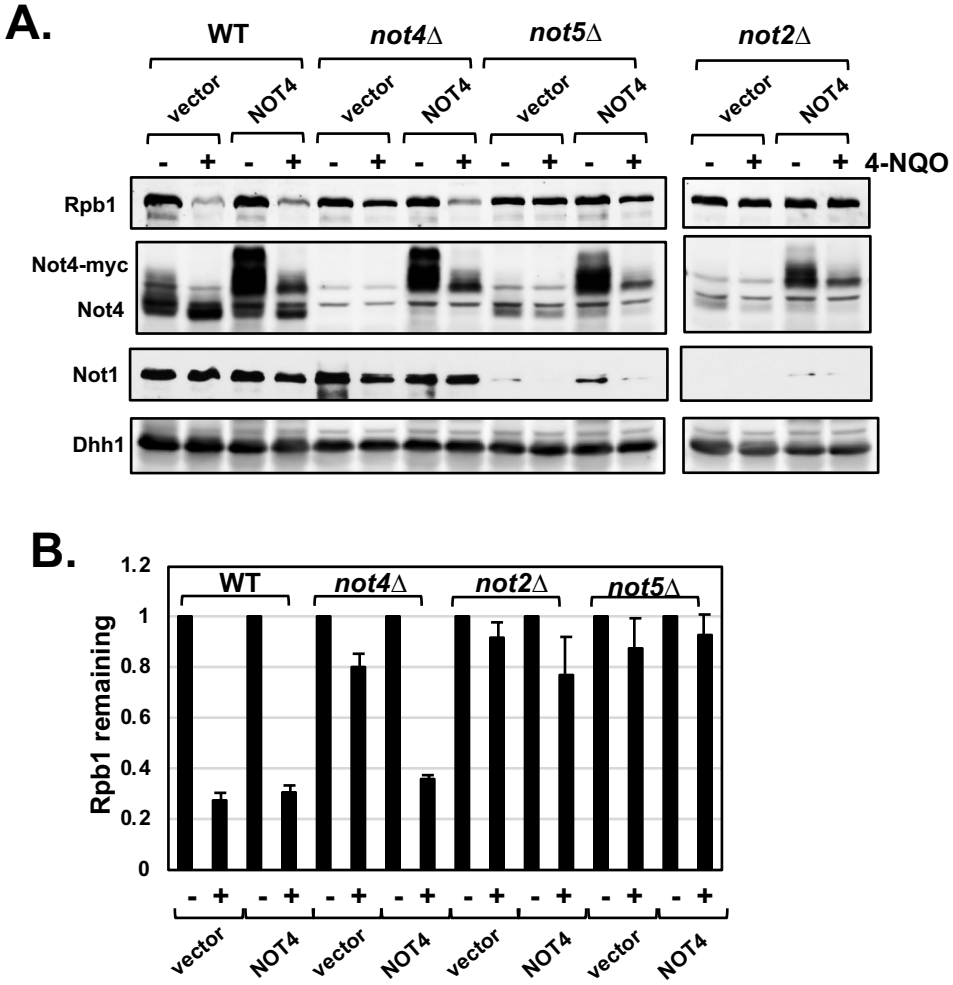


Figure S2. Overexpression of NOT4 does not suppress the Rpb1 degradation defect.
(A). Overexpression of *NOT4* does not suppress the Rpb1 degradation defect. WT*, not4*Δ*,not5*Δ and *not2*Δ cells were transformed with pRS425 or pRS425-*NOT4*-myc. Cells were treated with 5ug/ml 4-NQO and 100ug/ml cycloheximide for 90 min. Western blot for Rpb1, Not4 and Not1. The reduction in the overexpressed Not4 in treated cells is due to the instability of the excess Not4 protein compared to the Ccr4-Not complex, which was revealed by inhibiting ongoing protein synthesis. Not1 levels are strongly reduced in *not2*Δ and *not5*Δ cells, which could be weakly suppressed by overexpressing *NOT4*. (B). Quantification of Rpb1 turnover in *NOT4* overexpressing cells. The fraction of Rpb1 remaining was calculated, setting the untreated value (t=0) at 1.0. The Rpb1 signal was normalized to the loading control. Each data point represents the mean and standard deviation (N=3). (C). Overexpressing *NOT4* imparts a slight growth advantage to *not5*Δ and *not2*Δ mutants. Spot test for cell growth. Plates were scanned 44- and 70-hrs after plating.

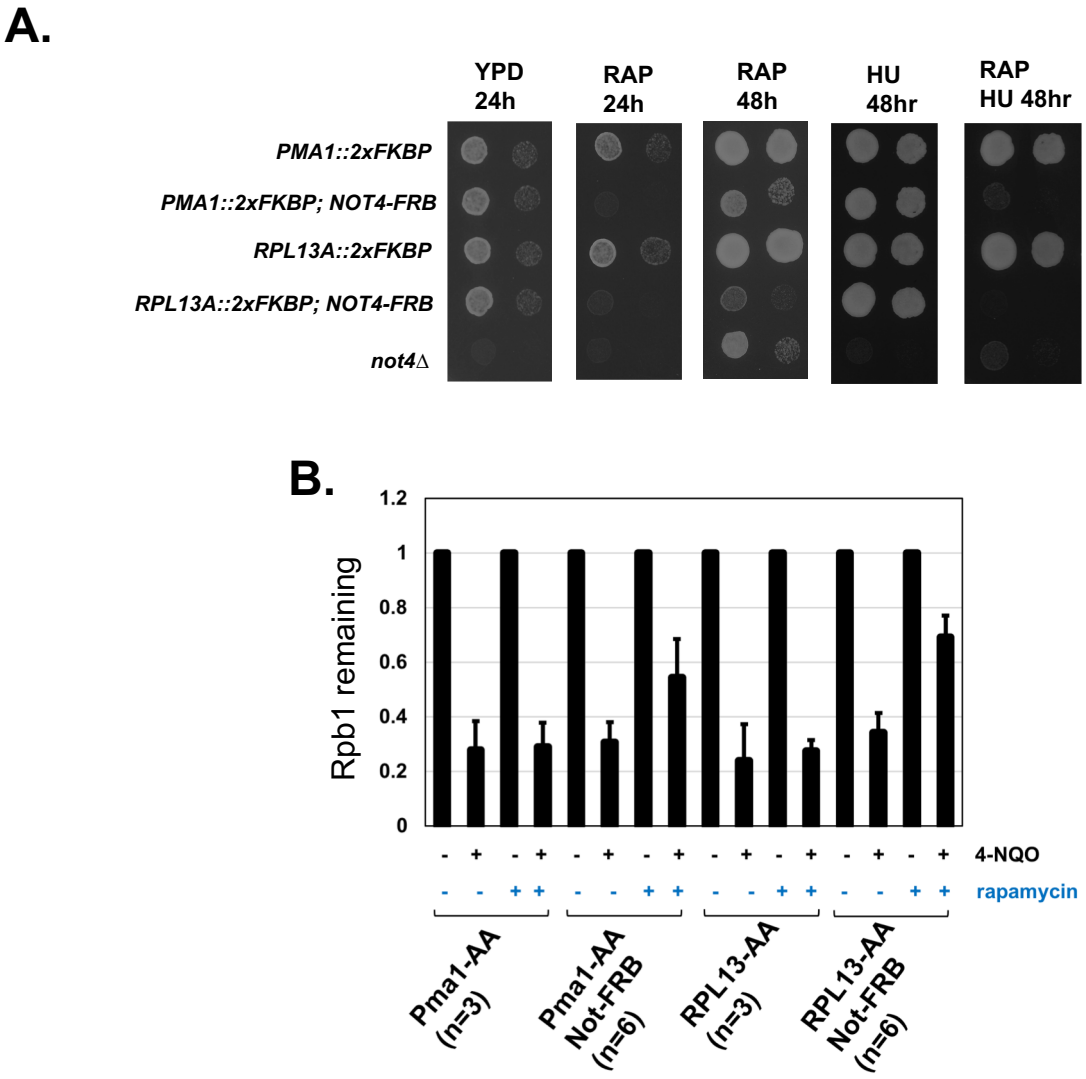
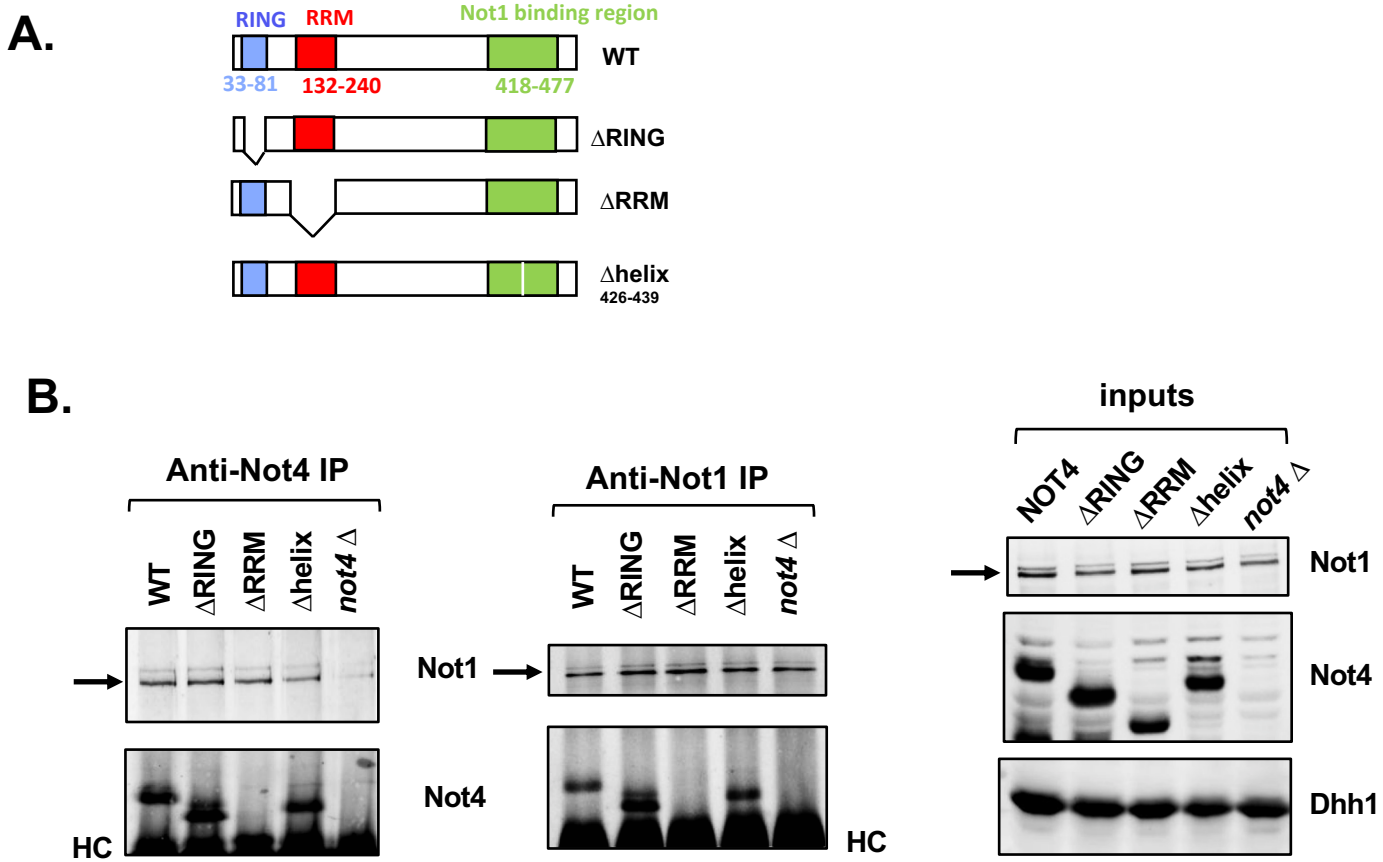


Figure S3. **Analysis of Anchor away strains.** (A). Spot growth test of anchor-away strains. Strains were spotted onto the media indicated in the panel. RAP plates were supplemented with 1ug/ml rapamycin. The *not4*Δ strain was constructed in the anchor-away strain (tor1-1, fpr1Δ). Hydroxyurea (HU) was added to 50 mM. (B). Quantification of Rpb1 degradation. Rpb1 signals were calculated as described in the legend of figure 1. Three independent experiments were quantified, two separate isolates of the Not4 AA strain was analyzed in each experiment. The values are the means and standard deviations.



Note: ΔRRM Not4 co-migrates with heavy chain

Figure S4. RING and RRM mutants associate with the Ccr4-Not scaffold protein Not1. (A). Schematic of mutants. The Δhelix mutant deletes amino acids that comprise one of the contacts with Not1. The mutation was not sufficient to disrupt the interaction. (B). Immunoprecipitation of the Ccr4-Not complex. Cell extract was immunoprecipitated using Not1 or Not4 antiserum and the bound proteins were detected by Western blotting. Note that the ΔRRM Not4 protein co-migrates with heavy chain in the IP samples. The arrow in the panels indicates migration of Not1, which is the lower band in the images.

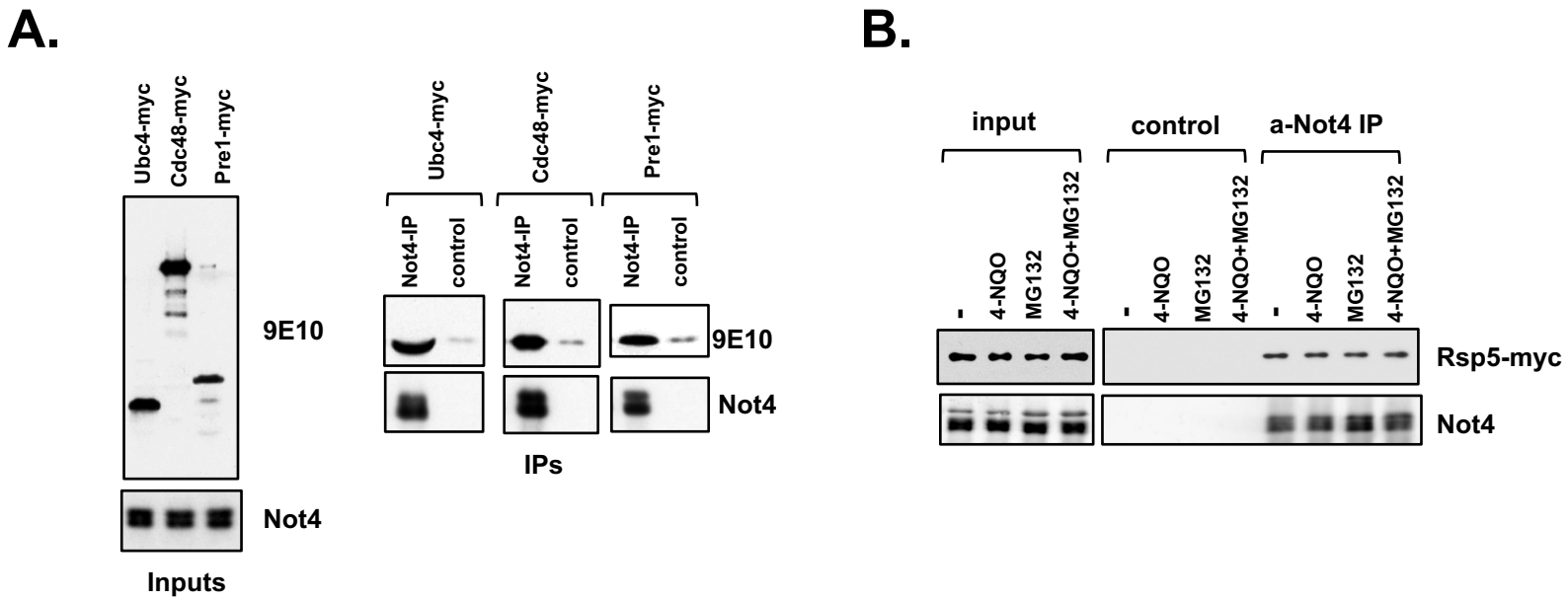
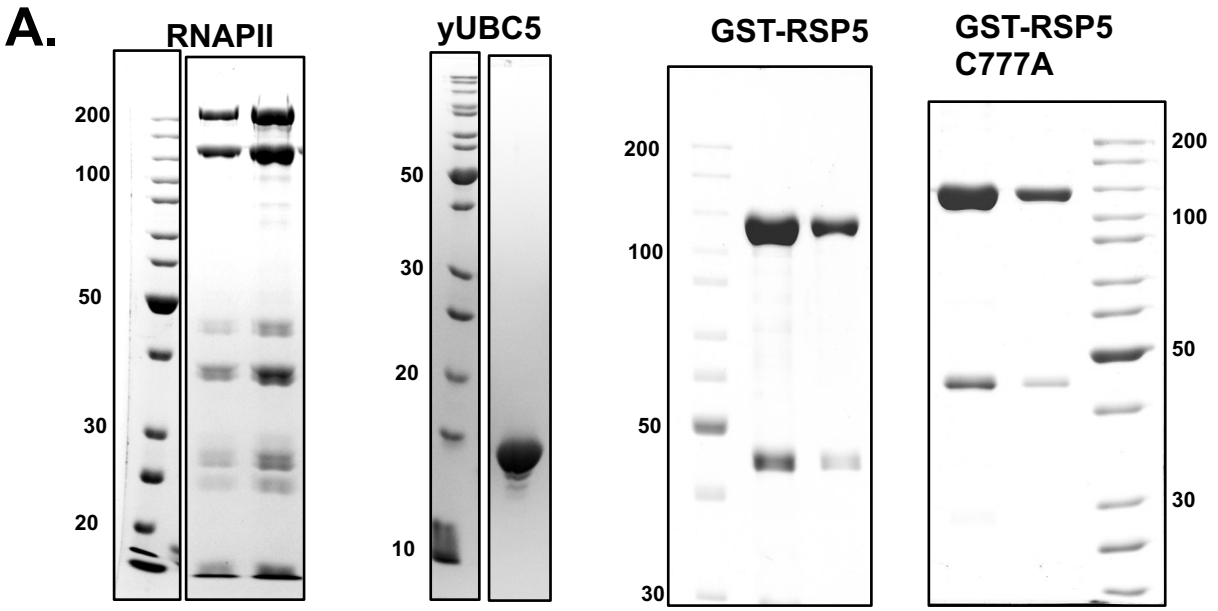
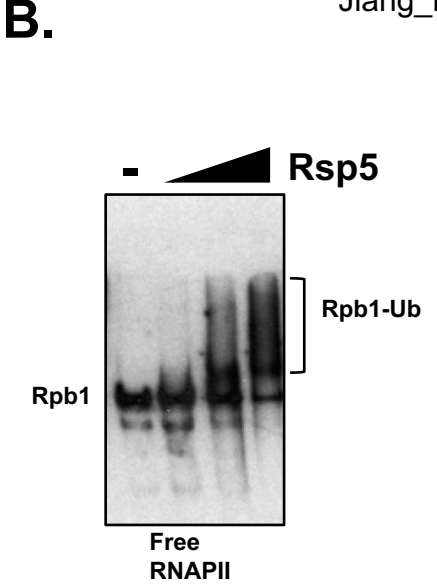
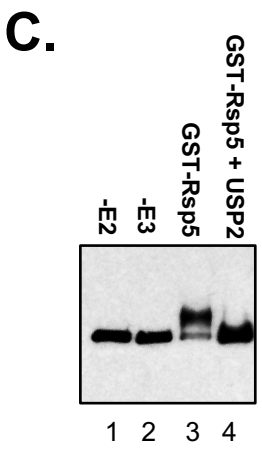


Figure S5. Not4 associates with factors involved in Rpb1 degradation. (A). Whole cell extract (input) was prepared from strains containing Myc-tagged Ubc4, Cdc48 and Pre1 and used in immunoprecipitation. Ccr4-Not was immunoprecipitated using Not4 antiserum. The left shows the input samples probed with anti-myc or anti-Not4 antibody. The right shows the Not4 immunoprecipitates probed with anti-myc and anti-Not4 antibody. No antibody protein A beads was used as control. (B). Co-immunoprecipitation of Not4 and Rsp5-myc in treated cells. Cells were treated with 5ug/ml 4-NQO for 30 min (4-NQO), 50 uM MG132 for 2h (MG132) or MG132 for 90 min and then 4-NQO for 30 min (4-NQO+MG132). Immunoprecipitation was carried out as described in panel A.







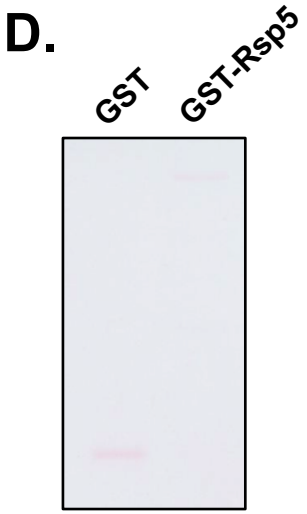


Figure S6. Purified proteins and Rpb1 ubiquitylation assay. (A). Coomassie blue stained gels of purified RNAPII and recombinant ubiquitylation enzymes. (B). Rsp5-dependent ubiquitylation assay. 300ng free RNAPII was incubated with E1 (50ng GST-Ube1), E2 (25ng UbcH5c) and recombinant Rsp5 (0ng, 25ng, 50ng and 100ng) for 60 min at 30 °C. Rpb1 was probed with 8WG16 antibody. (C). *In vitro* ubiquitylation of Rpb1 using GST-Rsp5. 20ul from the reaction mixture was collected after one-hour incubation at 30°C (lane 3). USP2 was then added to the mixture and further incubated 30 minutes at 30°C before running on a gel (lane 4). (D). Ponceau S stain of the blot of GST pull-down assay shown in figure 5C.

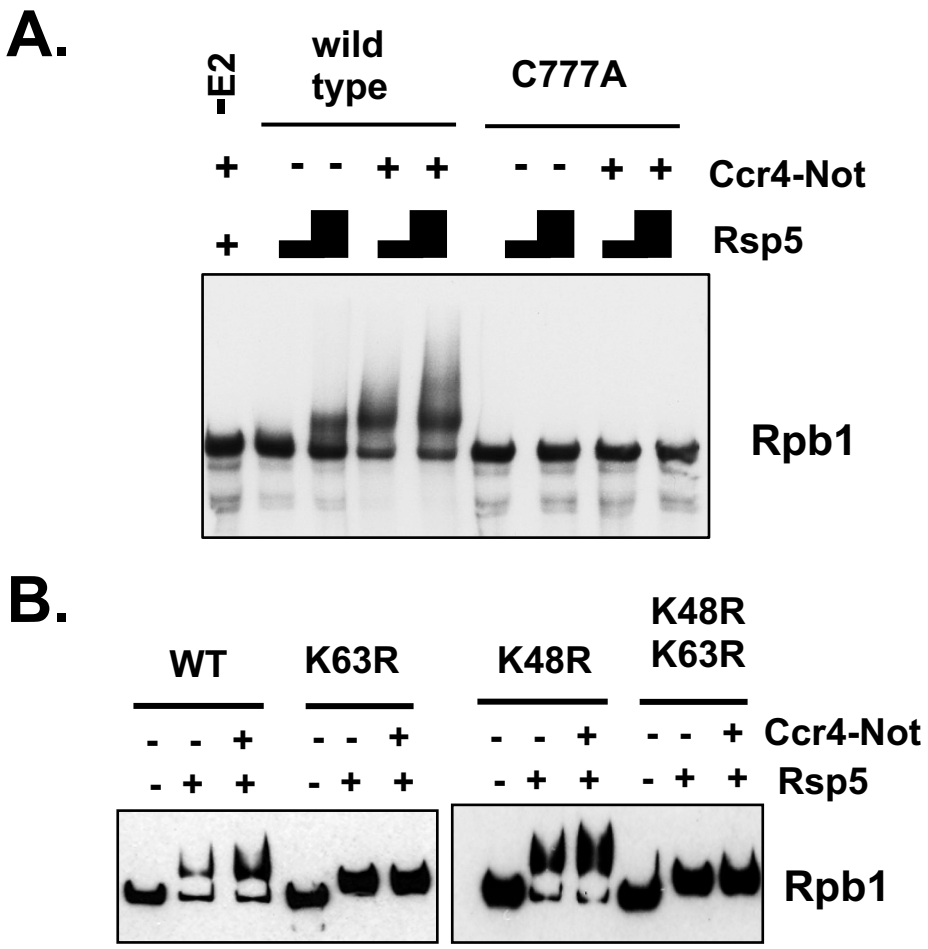


Figure S7. Rsp5 carries out ubiquitylation of Rpb1 in the presence of Ccr4-Not. (A). Ubiquitylation assay with and without Ccr4-Not using either wild type or a catalytic mutant (C777A) of Rsp5. 300ng free RNAPII was incubated with E1 (50ng GST-Ube1), E2 (50ng UbcH5c) and titrated amount of recombinant Rsp5 (50ng and 100ng), 8ug ubiquitin for 60 min at 30 °C. First lane is the control without E2. Rpb1 was probed with 8WG16 antibody. (B). Ubiquitylation reaction with ubiquitin substitutions. Reaction was done in the same condition as panel A except 4ug ubiquitin substitutions and 100ng recombinant GST-Rsp5 or 1ug purified Ccr4-Not complex were added where indicated.

Supplement Jiang et al.

Table S1. Strains used in this study

BY4741		MAT a his3Δ1; leu2Δ0; met15Δ0; ura3Δ0
BY4742		MAT alpha his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0
JR1179	BY4741	ccr4Δ::kanMX
JR1180	BY4741	caf1Δ:: kanMX
JR1911	BY4741	not2Δ:: kanMX
JR1912	BY4742	not2Δ:: kanMX
JR1181	BY4741	not5Δ:: kanMX
JR1916	BY4741	not5Δ:: kanMX
JR1188	BY4741	not3Δ:: kanMX
JR1189	BY4741	not4Δ:: kanMX
JR1531	BY4742	not4Δ::natMX
JR1873	BY4741	not4 ΔRING (Δ33-81)
JR1874	BY4741	not4 ΔRRM (Δ132-240)
JR1900	BY4741	not4 L35A
JR1901	BY4741	not4 L35A,I37A
JR1421	BY4741	caf40Δ:: kanMX
JR1532	BY4742	caf40Δ::natMX
JR1698	BY4742	rad26Δ::kanMX
JR1772	BY4742	not4Δ::natMX; rad26Δ:: kanMX
JR1696	BY4742	def1Δ::kanMX
JR1819	BY4742	not4Δ::natMX; def1Δ::HIS3 [pRS316-NOT4]
JR1742	BY4742	pdr5Δ::kanMX
JR1753	BY4742	pdr5Δ:: kanMX; not4Δ::natMX
JR1850	BY4742	pdr5Δ::URA3; RSP5-13MYC:: kanMX
JR1852	BY4742	pdr5Δ::URA3; UBC4-13MYC:: kanMX
JR1854	BY4742	pdr5Δ::URA3; CDC48-13MYC:: kanMX
JR1856	BY4742	pdr5Δ::URA3; PRE1-13MYC:: kanMX
JR1921		MAT alpha tor1-1 fpr1::natMX; PMA1-2×FKBP12::TRP1; Not4-FRB:: kanMX ade2-11; his3-11,15; leu2-3,112; ura3-1; trp1-1; can1-100
JR1922		MAT alpha tor1-1 fpr1::nat::MX; RPL13A-2×FKBP12::TRP1; Not4-FRB:: kanMX ade2-11; his3-11,15; leu2-3,112; ura3-1; trp1-1; can1-100
JR1408	BY4741	NOT4-TAP::HIS3, dst1Δ::URA3
JR1522	BY4741	NOT1-TAP::HIS3, dst1Δ::URA3
JR1413		MATa ade2-11; his3-11,15; leu2-3,112; ura3-1; trp1-1; can1-100 Rpb4-TAP::TRP1; pep4Δ::HIS3; prb1Δ::LEU2; prc1Δ::HISG
JR1868	BY4741	NOT1-TAP::HIS3; not4 ΔRING (33-81)

Supplemental Methods:

Not4 mutant construction by CRISPR-Cas9

The guide RNA (gRNA) for each mutant was designed using DESKGEN (https://www.deskgen.com/landing/) and cloned into pML104 plasmid (Laughery et al. 2015). The repair template was prepared by annealing two overlapping oligonucleotides and filling in with the Klenow fragment of DNA polymerase. In brief, 5ul 10uM of sense and anti-sense oligos were added into 5ul 10x buffer (300mM Tris-HCl (pH 7.8 at 25°C), 100mM MgCl2, 100mM DTT and 10mM ATP), 1ul 10mM dNTP and 31.5ul ddH2O. The mixture was heated at 95 degree for 10mins and cool down at room temperature for about 40 minutes. 7.5 units of Klenow polymerase was added afterwards and incubated at 25 degree for 20mins. Then reaction was stopped by adding 1ul of 0.5M EDTA and incubate at 75 degree for 20mins. The product was verified on agarose gels, and gel purification was performed when necessary. The sgRNA-containing plasmid and the corresponding repair template were co-transformed into BY4741 cells and selected on DEX/-URA plates. The plasmid was removed after gene editing by selection on 5-FOA plates. Mutants were verified by sequencing PCR-generated products and western blotting. Sequences of gRNA are as follows: Not4ΔRING: TGACAAATTTGATAACCACA; Not4ΔRRM: ATATTTTTCAGATTTCAGAG;
Not4 L35A or Not4 L35A, I37A: CCATTGGTTCAATACAAAGA
Sequences of repair templates:
Not4ΔRING
sense:
TTTGCAAGCAATCCACAACGCCTTAAGCAATTTTGATACGTCATTTTTATCGGAGGATGA
AGAAGATTATGATGACGAGA

anti-sense:

TAGCGAGCTTGGCTCTCTCCATTTTTAACTCCTCCGGAGATAATGTGACGTATCTGACGT

TCTCGTCATCATAATCTTCT

Not4ΔRRM

Sense:

AGAAAGAAAGCATAGAGAAAAAGAACGTAAAGAGAATGAATATACGAATAGGAAACATTT

ATCTGGTACCGGATTGCCAT

anti-Sense:

TATTAAAAGAATCAGCTTCTTCACCAGGTTCATGCAAAAACATACAGTTGGGATTTGGGC

ATGGCAATCCGGTACCAGAT

Not4 L35A

Sense:

CGCCTTAAGCAATTTTGATACGTCATTTTTATCGGAGGATGAAGAAGATTATTGTCCTGC

TTGTATTGAA

Anti-Sense:

GATAACCACAGGGACAAGGAAAAAAATTTTTATCAGTAATATCCATTGGTTCAATACAAG

CAGGACAATA

Not4 L35A, I37A

Sense:

CGCCTTAAGCAATTTTGATACGTCATTTTTATCGGAGGATGAAGAAGATTATTGTCCTGC

TTGTGCTGAA

Anti-Sense:

GATAACCACAGGGACAAGGAAAAAAATTTTTATCAGTAATATCCATTGGTTCAGCACAAG

CAGGACAATA

RNAPII purification:

RNAPII was purified from YJR1413. Twelve liters of cells were grown to saturation in 1.5X YP supplemented with 20 ug/ml adenine sulphate and 4% dextrose, harvested and resuspended in 1/5 a volume of 5X TEZ (250 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 uM $ZnCl_2$, 10 mM DTT, 50% glycerol), supplemented with 4 ug/ml Leupeptin,
5 ug/ml pepstatin A, 2ug/ml aprotinin, 2 mM benzamidine-HCL, 1mM PMSF and 5% DMSO. Cells were broken by mixing in a beadbeater with a stainless steel chamber (Biospec, Battlesville OK). The crude lysate was diluted 5-fold with 1x TEZ+0.5M KoAc, and then centrifuged at 5500xg, and then again at 40,000 rpm for 60 minutes in a 45Ti rotor. The clarified lysate was passed through a 5 ml human IgG-sepharose (GE Lifesciences) column at 0.8 mls/min. The column was washed sequentially with 20-25 column volumes (CV) of 1xTEZ+0.2M KoAc, 1xTEZ+0.5M KoAc and then 1xTEZ+0.5M ammonium sulfate at 2 ml/ml. The column was washed with 10 CV Q buffer (25 mM Tris-HCl, pH 7.5, 0.1M ammonium sulfate, 1 mM EDTA, 10 uM $ZnCl_2$, 1 mM DTT and 10% Glycerol), and then the beads were incubated with 50 ug tobacco etch virus (TEV) protease in 3 mls of the same buffer overnight at 4˚C. The liquid was collected and the beads washed with 3, 2 ml washes of Q0.1M. Peak fractions were pooled and concentrated to 2 mls using a microfiltration concentrator, 100KDa MWCO (Vivaspin® 6 centrifugal concentrator, 100KDa MWCO). The sample was passed over a Hi-Trap SP column and 1 ml MonoQ column arranged in series. After washing with 10

CV Q0.1M, the SP column was removed and polymerase was eluted in a 15 ml gradient to Q0.65M. The peak fractions were pooled. The typical concentration was 1.5-2.2 mg/ml protein and the molarity of the fraction was approximately 0.34M.

Ccr4-Not purification

Ccr4-Not complex was purified from 6L of either JR1408 or JR1522, containing Not4-TAP or Not1-TAP strains with *DST1* deleted, respectively(Kruk et al. 2011). The purification procedure was similar to that of RNAPII, up to the IgG-binding step. The clarified lysate was passed through a 1.5 ml IgG-sepharose column at 0.3-0.4 mls/min. The column was washed sequentially with 50ml of 1xTEZ+0.2M KoAc, 30ml of 1xTEZ+0.5M KoAc and then 5ml 1xTEZ+0.5M ammonium sulfate at ~1 ml/ml. The column was washed with 10ml TEV cleavage buffer (25 mM Tris-base, pH 7.5, 0.2M NaCl, 1mM $MgCl_2$, 1mM imidazole, 0.5 mM EDTA, 10% Glycerol, 0.01% NP40, 1mM DTT) and incubated with 15 ug TEV protease in 0.5 ml of the same buffer overnight at 4˚C. The liquid was collected and the beads washed with 3, 0.5 ml washes of TEV cleavage buffer. The collected liquid and washes were pooled and dialyzed for 2-3 hours in dialysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1mM $MgCl_2$, 2 mM TCEP and 0.001% NP40). The sample was concentrated to about 1-2mg/ml using a centrifugation device (Vivaspin® 6 centrifugal concentrator, 100KDa MWCO).

Purification of recombinant Ubc5, Def1, Rsp5 and Dsk2

The coding sequence of *UBC5* was amplified by PCR and cloned into the BamHI and BsrG1 sites of pST50(Tan et al. 2005) using Inphusion cloning (Takarabio), placing an N-terminal

hexahistidine tag on the protein separated by a TEV protease site. Protein was expressed in Rosetta2 cells in Luria broth by inducing with 0.5 mM IPTG for 16 hrs at 16˚C. Cells from 1L of culture were resuspended in lysis buffer (20 mM HEPES-OH pH 8.0, 500 mM NaCl, 10 uM $ZnCl_2$, 10 mM imidazole, 1.5 mM TCEP and 10% glycerol) supplemented with 0.5 mM PMSF, 1 mM benzamidine-HCl, 1 ug/ml pepstatin A, 0.2 ug/ml leupeptin and 0.2 ug/ml aprotinin. After a freeze-thaw cycle, the cells were lysed by sonication and clarified by centrifugation. The proteins were purified on Ni-agarose beads using standard procedures. Three milligrams of protein was digested for 2 hr at 23˚C with 150 ug TEV protease in 1 ml TEV digestion buffer (25 mM Tris-HCL, pH 7.5, 150 mM NaCl, 10 uM $ZnCl_2$, 1 mM imidazole, 0.1 mM EDTA, 1.5 mM TCEP and 10% glycerol). The undigested protein, 6HIS tag and TEV protease was removed by incubating the sample with Ni-NTA-agaorse beads.

The coding sequence of Def1-full length (FL) and Def1-500aa was amplified by PCR and cloned into the BamHI and BsrG1 sites of pST50. The purification procedure was same as that of Ubc5, except that the tag was not cleaved off the protein. GST-RSP5 was purified from pGEX-6P2-Rsp5 as described in a previous publication(Kus et al. 2005). GST-DSK2 was purified with the same procedure as GST-Rsp5, except that the protein was not eluted off the GSH beads. pGEX3-DSK2 was a generous gift of Dr. Jesper Q. Svejstrup.

Sequence of oligos for PCR were as follows.

UBC5 BamHI fwd: GTACTTCCAGGGATCCATGTCTTCCTCCAAGCGTATTG

UBC5 BsrG1 rev: ATCTGGATCTTGTACACGCTGAGGAAGGTAAGTCTAC

Def1 BamHI fwd: GTACTTCCAGGGATCCATGTCTACACAATTTAGGAAGTC

Def1-FL BsrG1 rev: ATCTGGATCTTGTACATTAGTAGAAACCTCTTGAATTTAGA

Def1-500aa BsrG1 rev: ATCTGGATCTTGTACACTATTGTTTTTGACTTTGTGGACTTTG

References:

Kruk JA, Dutta A, Fu J, Gilmour DS, Reese JC. 2011. The multifunctional Ccr4–Not complex directly promotes transcription elongation. *GENES & DEVELOPMENT* **25**: 581-593.

Kus B, Gajadhar A, Stanger K, Cho R, Sun W, Rouleau N, Lee T, Chan D, Wolting C, Edwards A et al. 2005. A high throughput screen to identify substrates for the ubiquitin ligase Rsp5. *Journal of Biological Chemistry* **280**: 29470-29478.

Laughery MF, Hunter T, Brown A, Hoopes J, Ostbye T, Shumaker T, Wyrick JJ. 2015. New vectors for simple and streamlined CRISPR-Cas9 genome editing in Saccharomyces cerevisiae. *Yeast* **32**: 711-720.

Tan S, Kern RC, Selleck W. 2005. The pST44 polycistronic expression system for producing protein complexes in Eseherichia coli. *Protein Expression and Purification* **40**: 385-395.