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Supplemental Data

Distinct Modes of Regulation of the Uch37 Deubiquitinating Enzyme in the Proteasome and in the Ino80 Chromatin Remodeling Complex

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Supplemental Experimental Procedures

Yeast two-hybrid assays

Yeast two-hybrid assays employed the same system described in Yao et al., 2006, except that the prey constructs were Ino80 complex subunits Ino80, Tip49a, Tip49b, NFRKB, Arp5, Arp8, Baf53a, Ies2, Ies6, YY1, MCRS1, FLJ20309, and CCDC95.

Antibodies and affinity purifications

Whole cell extracts and cytoplasmic and nuclear extracts of HEK293 cells, and whole cell extracts of Sf21 insect cells were prepared as described (Yao et al., 2006).

Anti-Flag (M2), anti-Flag (M2) agarose, rabbit polyclonal anti-HA antibody, mouse anti-HA antibody (HA-7), mouse anti-His antibody, mouse anti-alpha tubulin antibody and anti-HA agarose were obtained from Sigma; anti-Myc agarose was obtained from Santa Cruz; monoclonal anti-GST antibody was obtained from Covance; anti-S1 antibody was obtained from Boston Biochem (Boston, MA). Anti-Uch37, anti-hRpn13, and anti-Ies2 antibodies have been described previously (Cai et al., 2007; Yao et al., 2006). Rabbit polyclonal anti-NFRKB antibody was raised against recombinant NFRKB 1-101 that was expressed in E. coli (Cocalico Biologicals, Reamstown, PA).

Recombinant proteins

Expression and purification of His10-hRpn13 (also known as Adrm1), Flag-hRpn13, GST-Uch37 and GST-UCH (N terminal 1-237 amino acid of Uch37) have been described (Yao et al., 2006). To express NFRKB 1-101, a pET19b plasmid encoding NFRKB 1-101 with an N-terminal 10x histidine tag, and a pET41a plasmid encoding Uch37 with an N-terminal GST tag, were co-transformed into BL21 (DE3) codon plus E. coli. (Stratagene). Log phase cultures in LB medium supplemented with ampicilin, kanamycin, and chloramphenicol were induced with 0.1 mM IPTG at 16 °C overnight. From the cell pellet, NFRKB 1-101 was purified from the soluble fraction using Ni-NTA agarose (Qiagen) following the manufacturer's instructions. At this point, the predominant proteins that co-purified with N101 were fragments of Uch37, which were presumably produced as a result of bacterial proteolytic activities. Eluates from the Ni-NTA column were desalted to remove imidazole, and GuHCl was added to 6 M followed by incubation with Ni-NTA agarose again. Under this denaturing condition, only N101 remained bound. NFRKB 1-101 was refolded on the column by sequentially washing the

resin with buffers containing 50 mM NaPi, pH 8, 300 mM NaCl, 10 mM imidazole, and 4.8 M, 3.6 M, 2.4 M, 1.2 M, and 0 M GuHCl. After elution with 250 mM imidazole, N101 was further purified by gel filtration on a Superdex-75 column.

NFRKB fragments 1-101, 1-465, 449-1324,and full-length NFRKB were subcloned into pBacPAK8 with N-terminal 6x histidine-Flag tags ("HF") and expressed using the BacPAK baculovirus expression system (Clontech). Similarly, hRpn13 was subcloned into pBacPAK8 with N-terminal 6x histidine-Myc tags ("HM") and expressed using the baculovirus expression system. HF-N465 was isolated from infected Sf21 cell lysates by anti-flag affinity purification followed by Ni-NTA agarose. ATP (5 mM) was included in wash buffers to remove co-purifying Hsc70 proteins. HM-hRpn13 was purified from infected Sf21 cell lysates by Ni-NTA agarose followed by immobilization on anit-Myc agarose. NFRKB fragments 1-101, 102-227, 228-352, and 353-465 were labeled with ^{35}S-methionine by *in vitro* transcription and translation using the EcoProT7 system (Novagen).

Mass Spectrometry

Proteins are identified using a modification of the multidimensional protein identification (MudPIT) procedure (Washburn et al., 2001; Wolters et al., 2001). Numbers shown in Figure 1B are NSAF (Normalized Spectral count Abundance Factor) (Zybailov et al., 2006). To account for the fact that larger proteins tend to contribute more peptides and thus more spectra, spectral counts are divided by protein length (SAF). SAF values are then normalized against the sum of all SAFs for a particular sample (removing redundant proteins) to allow comparison of protein levels across different samples using NSAF values. NSAF for a protein is calculated as follows: NSAF = (Spectral Count / Length) /Σ(Spectral Count / Length) for all non-redundant proteins in run. The MS-MS datasets were searched using SEQUEST against a database combining the following proteins: 40877 human sequences (NCBI 2005-12-08 release), 102 "custom" sequences corresponding to various epitope tagged human proteins, 58794 mouse sequences (NCBI 2005-06-08 release), 22725 rat sequences (NCBI 2006_03-20 release), 21737 chimpanzee sequences (NCBI 2006_03-20 release), 35907 cow sequences (NCBI 2005-10-06 release), 36 human adenovirus 5 sequences (NCBI 2005-06-08 release), and 177 sequences from common contaminants, including human keratins, IgGs, and proteolytic enzymes. Note that although the MS/MS datasets were searched against sequences from different organisms, only human proteins are reported here. To estimate false positive discovery rates (FDR), each sequence was randomized (keeping the same amino acid composition and length), and the resulting "shuffled" sequences were added to the database listed above and searched at the same time. The total number of sequences searched was 360622. Spectra-to-peptide matches were retained only if they were to full tryptic peptides of at least 7 amino acids and had a normalized difference in cross-correlation scores of at least 0.08; minimum cross-correlation scores of 1.8 for +1, 2.0 for +2, and 3.0 for +3 spectra; and a maximum Sp score of 10. No spectra matched to shuffled peptides passed the selection criteria used to establish the protein list, yielding a FDR of 0%. The control protein list was derived from 7 independent FLAG immunopurifications from nuclear extracts and S100 fractions of parental HEK293(FRT) cells.

Supplemental References

Cai, Y., Jin, J., Yao, T., Gottschalk, A.J., Swanson, S.K., Wu, S., Shi, Y., Washburn, M.P., Florens, L., Conaway, R.C., *et al.* (2007). YY1 functions with INO80 to activate transcription. Nat Struct Mol Biol *14*, 872-874.

Washburn, M.P., Wolters, D., and Yates, J.R., 3rd (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol *19*, 242-247.

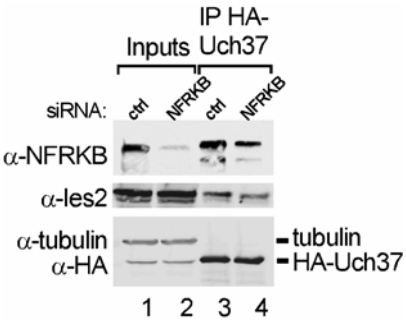
Wolters, D.A., Washburn, M.P., and Yates, J.R., 3rd (2001). An automated multidimensional protein identification technology for shotgun proteomics. Anal Chem *73*, 5683-5690.

Yao, T., Song, L., Xu, W., DeMartino, G.N., Florens, L., Swanson, S.K., Washburn, M.P., Conaway, R.C., Conaway, J.W., and Cohen, R.E. (2006). Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1. Nat Cell Biol *8*, 994-1002.

Zybailov, B., Mosley, A.L., Sardiu, M.E., Coleman, M.K., Florens, L., and Washburn, M.P. (2006). Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae. J Proteome Res *5*, 2339-2347.

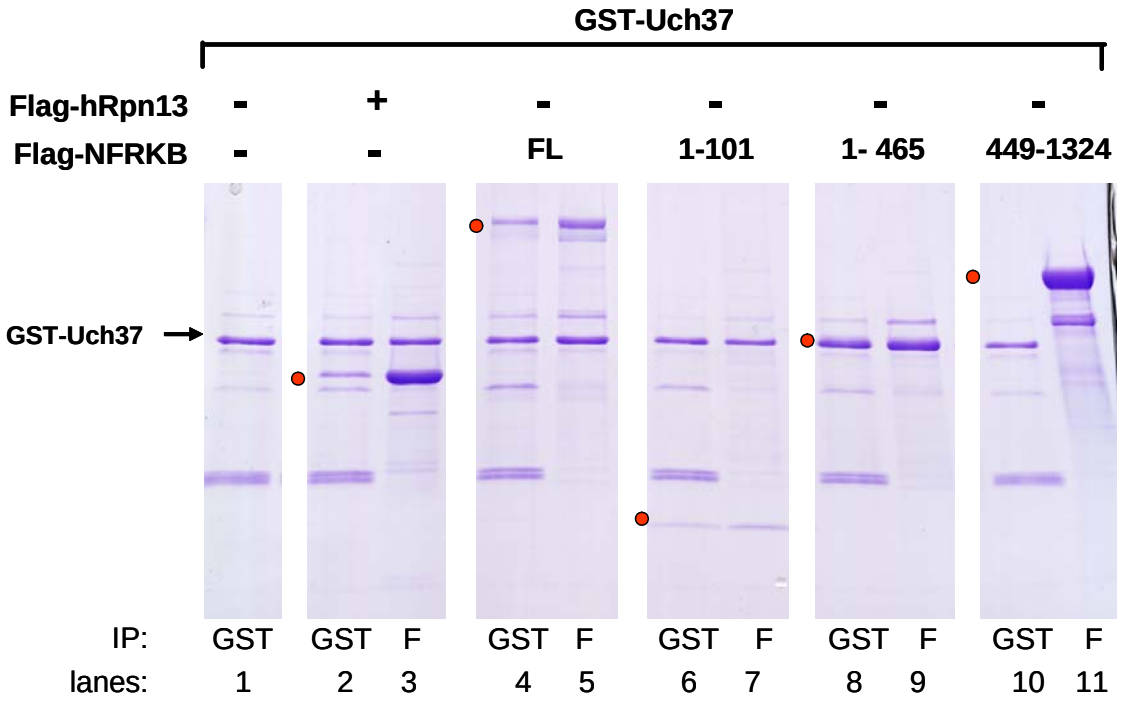
Supplemental Figures

Figure S1.



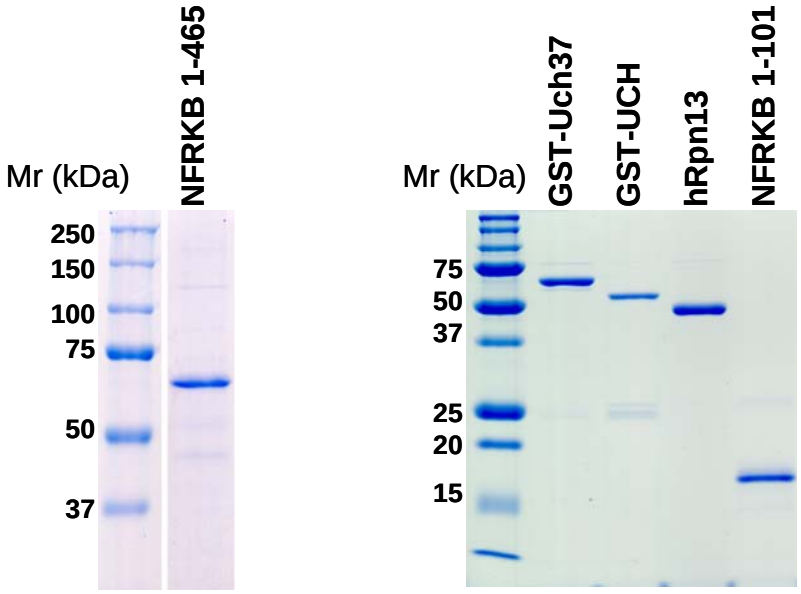
HEK293 cells stably expressing 2XHA-Uch37 were transfected with either control or NFRKB siRNAs. Uch37 and associated proteins were immunoprecipitated via the HA tag, analyzed by immunoblotting with anti-NFRKB, anti-HA, anti-Ies2, or anti-tubulin antibodies.

Figure S2.



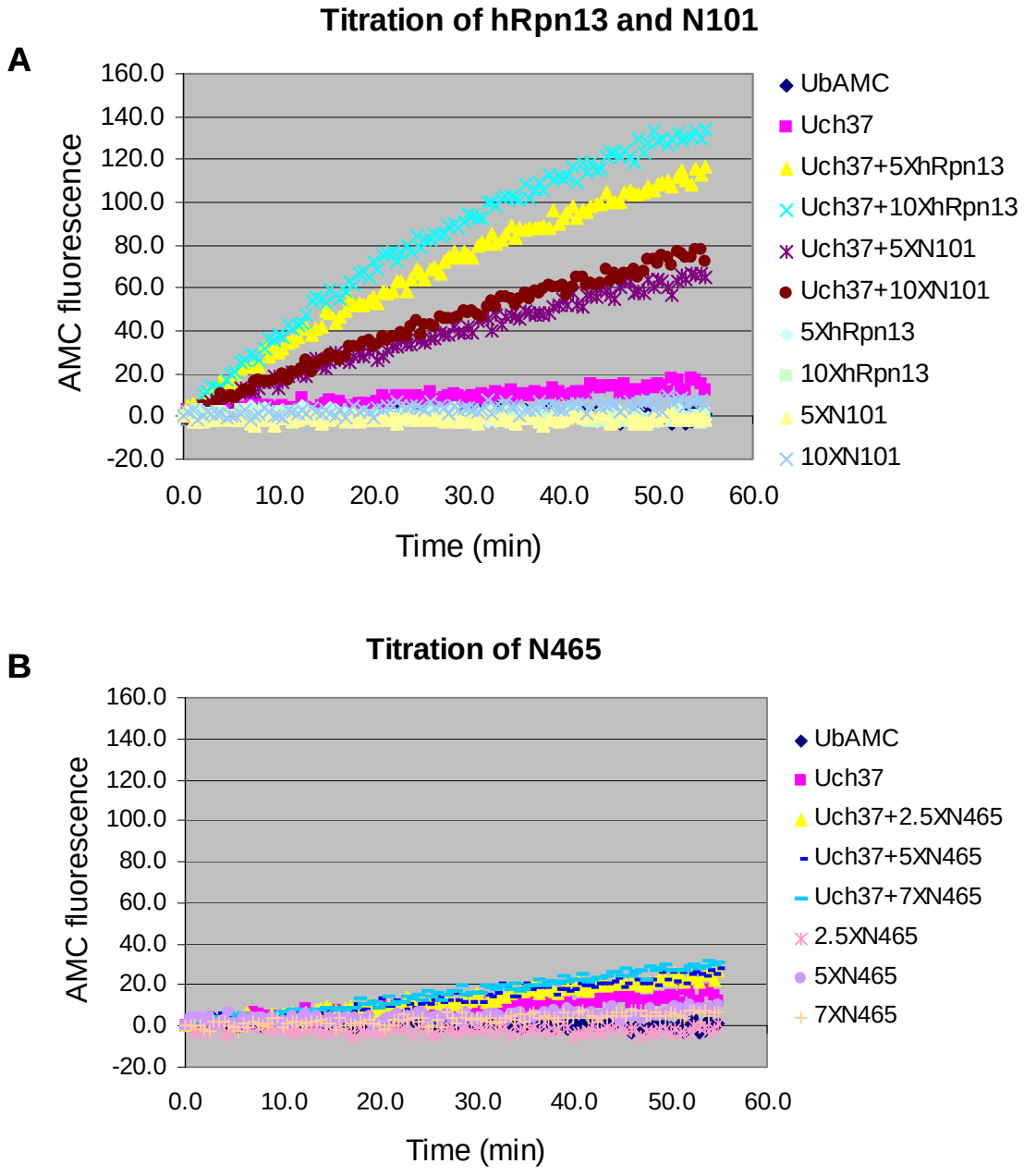
NFRKB 1-101 is necessary and sufficient for association with Uch37. GST-Uch37 was expressed by itself in insect cells and isolated using glutathione-agarose (lane 1) or co-expressed with its Flag-tagged binding partners (lanes 2-11). In the latter cases, reciprocal pull-downs used glutathione-agarose (GST) or anti-Flag agarose (F). All eluates were analyzed by SDS-PAGE and coomassie staining. The filled circles indicate positions of proteins co-expressed with GST-Uch37. Note that NFRKB 1-465 overlaps with GST-Uch37 on the gel.

Figure S3.



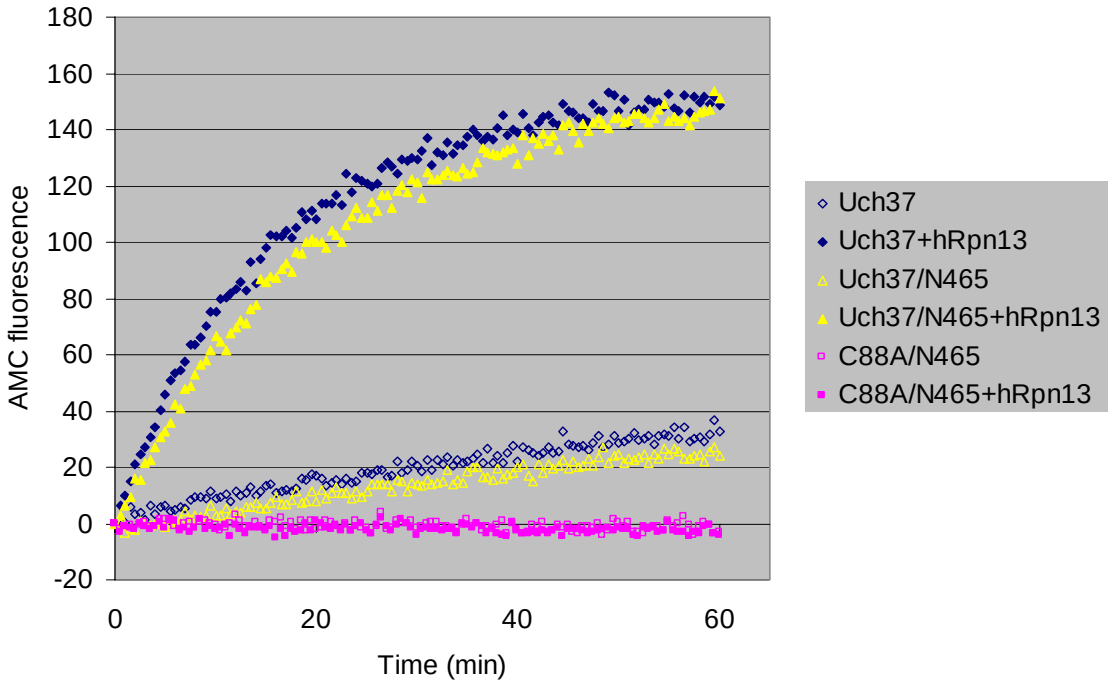
SDS-PAGE analysis of purified recombinant proteins. 0.5 µg of purified recombinant GST-UCH or 1 µg of each of the other indicated proteins was analyzed by SDS-PAGE and coomassie staining.

Figure S4.



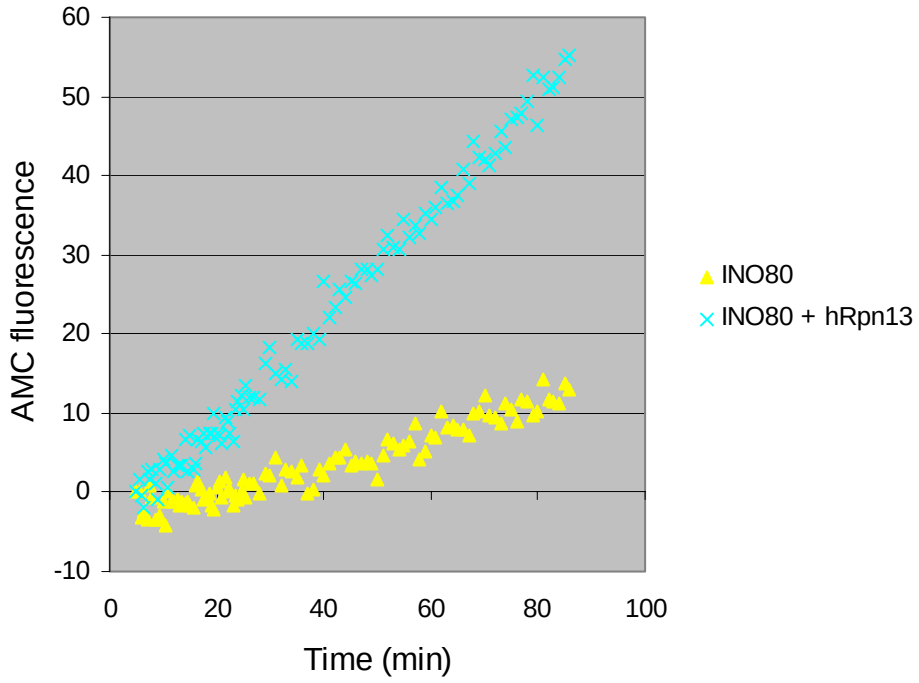
Titration of Uch37 with its binding partners. 2.5 nM of GST-Uch37 were used in each assay as described in METHODS. Indicated amounts of Uch37 binding partners (molar ratio) were present in each assay.

Figure S5.



NFRKB 1-465 does not repress UbAMC hydrolysis by Uch37. 10 nM GST-Uch37 or GST-Uch37/Flag-N465 complex, with or without 50 nM hRpn13, were incubated with 0.5 µM UbAMC at 30 °C. GST-Uch37/Flag-N465 complex was isolated via the GST and the Flag tags by sequential affinity purification from insect cells that co-expressed GST-Uch37 and Flag-N465. To confirm that the purified complex is free of contaminating DUBs, the active-site mutant GST-Uch37 (C88A) in complex with Flag-N465 were purified and assayed in parallel.

Figure S6.



Immobilized hINO80 can be activated by hRpn13. hINO80 immobilized on anti-Flag-agarose was incubated with 0.5 µM UbAMC with or without recombinant hRpn13. AMC fluorescence is monitored continuously at 30 °C by a SepctraMAX plate reader that was programed to shake in between readings.