SUPPLEMENTARY MATERIAL

Protein-linked ubiquitin chain structure restricts activity of deubiquitinating enzymes

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Running title: *Ubiquitin chain structure restricts DUB activity*

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Table S1. Apparent initial rates of substrate deubiquitination were estimated for each experiment from the slope of the initial linear phase of substrate degradation, with protein amounts expressed as arbitrary units based on quantitation of band intensity from storage phosphor screens. Ubp12 and Ubp15 were recombinant unless denoted "Yeast." Concentrations of enzyme and initial substrate are shown, along with the ubiquitin (or linkage, i.e. K63 denotes K63-linked chains). In cases where substrate was too dilute for accurate measurement, an upper limit of the concentration was estimated. The final three columns show the fold-increase of rates of monoubiquitin hydrolysis as compared to di-, tri- and tetraubiquitin.

						Apparent Initial Rates (arbitrary units)								Relative Rates		
Fig.	DUB	conc (nM)	substrate	sub conc (nM)	Ub	mono	mono error	di	di error	tri	tri error	tetra	tetra error	mono / tetra	mono / tri	mono / di
3D	Ubp15	0.033	Ub-AMC	2500		2 E+0	2 E-2									
3E	Ubp15	4	Pds1	<0.2		6 E-2	2 E-3	4 E-2	5 E-3	2 E-2	2 E-3	8 E-3	1 E-3	7	4	1
3E	Ubp15 (Yeast)	10	Pds1	<0.2		1 E-1	1 E-2	3 E-2	5 E-3							3
3G	USP7	6	CycB	<0.2		2 E-1	3 E-2	4 E-2	5 E-3	1 E-2	3 E-3	7 E-3	9 E-4	29	14	5
3G	Ubp15	130	CycB	<0.2		6 E-2	5 E-3	3 E-2	3 E-3	5 E-3	2 E-3	1 E-2	2 E-3	6	11	2
4A	Ubp15	155	Pds1	<0.1		4 E-1	6 E-2					3 E-2	4 E-3	16		
4A	Ubp15ΔC	155	Pds1	<0.1		7 E-2	8 E-3					2 E-2	3 E-3	3		
4A	Ubp15 ΔN	155	Pds1	<0.1		6 E-2	9 E-3					2 E-2	3 E-3	3		
4D	Ubp15	0.05	Ub-AMC			5 E+3	1 E+2									
4D	Ubp15ΔC	0.05	Ub-AMC			5 E+2	7 E+0									
4D	Ubp15 ΔN	0.05	Ub-AMC			1 E+3	1 E+1									
4D	Ubp15 ΔC ΔN	0.05	Ub-AMC			7 E+1	5 E+0									
5C	Ubp12	0.033	Ub-AMC	2500		1 E+0	2 E-2									
5G	Ubp15	87	Pds1	<0.4		7 E-1	2 E-1	4 E-1	1 E-1	1 E-1	2 E-2	3 E-2	3 E-3	21	5	2
5G	Ubp12	39	Pds1	<0.4		1 E-1	8 E-3	1 E-1	1 E-2	9 E-2	1 E-2	6 E-2	1 E-2	2	1	1
5J	Ubp15	87	CycB	<0.2		1 E-1	2 E-2	4 E-2	4 E-3	2 E-2	1 E-3	9 E-3	1 E-3	14	7	3
5J	Ubp12	39	CycB	<0.2		1 E-1	1 E-2	1 E-1	4 E-3	6 E-2	9 E-3	2 E-2	3 E-3	6	2	1
6A	Ubp15	0.44	CycB	<0.1	WT	4 E-1	3 E-2	4 E-2	4 E-3							10
6A	Ubp12	0.5	CycB	<0.1	WT	2 E-1	7 E-3	2 E-2	1 E-3							9
6D	Ubp15	0.44	CycB	<0.1	76A	5 E-2	2 E-3									
6D	Ubp12	0.5	CycB	<0.1	76A	5 E-3	3 E-4									
7A	Ubp15	4.4	CycB	<0.4	I44A	4 E-1	4 E-2	2 E-1	1 E-2	2 E-1	9 E-3				2	2
7B	Ubp15	0.35	CycB	6	K63	1 E-1	6 E-3					4 E-2	2 E-3	4		
7C	Ubp12	20	CycB	<0.4	I44A	4 E-1	2 E-2	7 E-1	5 E-2	8 E-1	9 E-2				0.6	0.6
7D	Ubp12	0.4	CycB	6	K63	6 E-2	2 E-3					2 E-2	2 E-3			

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Strains and Plasmids—Yeast strains are derived from W303 (yDOM90; *bar1::hisG ade2-1, can1-100, his3-11,15, leu2-3, trp1-1, ura3-1, ssd1-d*), with the exception of SDL145 (*ubp6::URA3 rpn11::RPN11-TEVProA (HIS3)*), which was a generous gift of D. Finley. DUBs were C-terminally TAP-tagged at their endogenous loci using established PCR-based protocols (1, 2).

Construction of Ubp12 and Ubp15 expression plasmids was carried out with standard techniques. Full-length *UBP12* and *UBP15* were PCR amplified from yeast genomic DNA with primers to add a C-terminal 6xHIS tag, then ligated into pGEX-6P-2 (GE Healthcare, Piscataway NJ) to add an N-terminal GST tag linked to the DUB by a PreScission protease site. N- and C-terminal deletion constructs of Ubp15 were made from this vector with the QuikChange site-directed mutagenesis kit (Stratagene). A GST-TEV-PKA-ubiquitin expression construct (pRDB1725) and a K48R derivative of this plasmid (pRDB1882) were generous gifts from R. Deshaies (3). These pGEX4t-1-based plasmids incorporate a cAMP-dependent protein kinase (PKA) phosphorylation site between the TEV site and the start methionine of ubiquitin. These plasmids were modified by QuikChange site-directed mutagenesis to separately incorporate I44A or G76A mutations. *PDS1-110* and a similar allele (*PDS1-zeroK*) in which arginine was encoded in place of all lysines were synthesized by GENEART (Regensburg, Germany) with codon optimization for expression in bacteria. To make expression constructs, both genes were subcloned into pET28a, incorporating N-terminal 6xHIS tags followed by TEV sites. QuikChange site-directed mutagenesis of *PDS1-zeroK* to restore the codons for K8 and K33 yielded the Pds1-K33 expression construct. *CycB-K60* was synthesized by Integrated DNA Technologies (Coralville, IA) with codon optimization for expression in bacteria. A GST-TEV-PKA expression construct was made by replacing ubiquitin in pRDB1725 (described above) with CycB-K60, incorporating a C-terminal 6xHIS tag before the stop codon. Expression constructs for human Ube1 and 10xHIS-Cdc34 were generous gifts of D. Komander (4, 5).

Synthesis of phosphorylated diubiquitin-CycB—The experiments in Figure 5I used diubiquitinated CycB-K60 in which only the distal ubiquitin was labeled. This substrate was made in several steps. First, K48-linked diubiquitin with distal PKA-K48R ubiquitin was synthesized in a Cdc34-dependent reaction essentially as described (4), using 2mM wild-type ubiquitin and 10 mM K48R ubiquitin bearing an N-terminal PKA site. A 1.5 ml ligation reaction contained 0.1 μM Ube1, 40 μM Cdc34, 2 mM ubiquitin (Boston Biochem) and 10 mM K48R ubiquitin (with an N-terminal PKA site as described above) in QAH8 (25 mM HEPES pH 7.8, 100 mM NaCl, 1 mM $MgCl_2$) with 4 mM ATP and 0.6 mM DTT. This reaction was incubated at 37°C overnight and diluted 30-fold in 50 mM ammonium acetate pH 4.5 with 10 mM 2-mercaptoethanol. The mixture of di-, tri- and oligoubiquitin chains was separated using MonoS cation exchange chromatography, eluting chains of different length over a shallow gradient of 0-1 M NaCl. Chains terminating with PKA-K48R ubiquitin eluted at significantly higher salt concentrations than wild-type chains of equivalent length. Purified diubiquitin was dialyzed to QAH8 and concentrated to approximately 10 mg/ml using Centriprep and Amicon Ultracel spin concentrators (Millipore). Diubiquitin was phosphorylated as described above. Ubc4 was charged for 20 min at room temperature with this phosphorylated diubiquitin and ligated to unlabeled CycB-K60 in a 6-hour APC/C reaction. The reaction was terminated by heating as above. The supernatant was diluted to 1x LDS sample buffer (6x stock = 1.2 M Triethanolamine, 6% Lithium dodecyl sulfate, 6% Ficoll-400, 60% glycerol, 0.0375% each of bromophenol blue, phenol red and Coomassie G250 brilliant blue) and separated on a 1.5 mm preparative 10% Tris/Tricine gel. The CycB-diubiquitin band was cut out of the gel, pulverized and incubated at room temperature with extraction buffer (10 mM HEPES pH 7.4, 100 mM NaCl). Serial extraction yielded approximately 5 ml (10 gel volumes), which was filtered through cotton, concentrated to 250 μl, re-diluted to 5 ml with extraction buffer and re-concentrated. Repeating the dilution and concentration again yielded 200 μl product at 225 nM.

SUPPLEMENTAL REFERENCES

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