Supplemental data

Stability of the endosomal scaffold LAMTOR3 depends on heterodimer assembly and proteasomal degradation*

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Legends of supplemental figures:

Supplemental Figure 1. Characterization of Myc_6LAMTOR3 stable clones. (A) Myc_6LAMTOR3*LAMTOR2*$^{-/-}$ and Myc_6LAMTOR3*LAMTOR2*$^{f/-}$ stable clones were harvested and lysed. Samples were loaded on 15% SDS-PAGE gels and Western blot membranes were probed with antibodies against the mentioned proteins. (B) Amino acid sequence of *Mus musculus* MAP2K1IP1 (LAMTOR3). Highlighted in Grey Bold Italic are methionine M1 and cysteine C73. (C) Growth curves of *LAMTOR2*$^{-/-}$, *LAMTOR2*$^{f/-}$ and Myc_6LAMTOR3*LAMTOR2*$^{-/-}$. $1x10^5$ cells of *LAMTOR2*$^{-/-}$, *LAMTOR2*$^{f/-}$and Myc_6LAMTOR3*LAMTOR2*$^{-/-}$ cells were plated into 10 cm dishes. Cells were counted using a Casy cell counter from Schärfe system GmbH at the indicated times (Hours). Graphic represents average of 3 independent experiments (M±STDEV, n=3).

Supplemental Figure 2. Calibration of Western blotting quantification using CCD camera. Quantifications of relative protein amounts after titration are shown in panels A and B. Different amounts of complex, corresponding to the mentioned amounts of LAMTOR3 were loaded on a SDS PAGE. Detection was performed using the CCD camera as described in Material and Methods. (C) His_6LAMTOR2LAMTOR3 complex was purified from *E. coli* to high purity. Membrane was probed with LAMTOR3 antibody.

Supplemental Figure 3. Velcade treatment of *LAMTOR2*$^{-/-}$ MEFs shifts LAMTOR3 to high molecular weight fractions (HMW). Size exclusion chromatography (SEC) from *LAMTOR2*$^{-/-}$, *LAMTOR2*$^{f/-}$ MEFs lysates treated with 100nM Velcade for 7 hours. Lysates were run on an Äkta HIPrep1660SephacrylS200HR column. Obtained 5ml fractions were precipitated using Trichloroacetic acid and Deoxycholate and resuspended in Laemmi sample buffer. Samples were separated by SDS-PAGE, analyzed by Western blotting and probed with the indicated antibodies.

Supplemental Figure 4. Recombinant LAMTOR3 is not degraded by the purified 20S proteasome. (A) Alignment of the Carboxy-terminus of human and mouse p21 with the Amino-terminus of LAMTOR3. Mouse and Human LAMTOR3 show 97% identity with no amino acid difference in the region here analyzed. (B) Purified 20S proteasome were incubated or not with Velcade for 10 minutes at 37ºC. Recombinant H_6LAMTOR2LAMTOR3, H_6LAMTOR3LAMTOR2 or H_6T7LAMTOR3 were purified from *E. coli*. P21 and p27 were in vitro transcribed and translated. Proteins were individually incubated with PBS alone, 20S subunits or 20S previously blocked by Velcade. Sample volume was adjusted and samples were incubated at 37ºC for the mentioned times. Reaction was stopped by addition of Laemmli sample buffer. Samples were separated by SDS-PAGE and probed with the indicated antibodies.

Supplemental Figure 5. The degradation of endogenous LAMTOR3 is lysosomal independent. (A) *LAMTOR2*$^{f/-}$ MEFs were treated with 100µg/ml E64, 200µM Leupeptin or 100 µM Chloroquine for 3 hours, harvested and lysed. Samples were loaded on 15% SDS-PAGE gels and Western blot membranes were probed with antibodies against the mentioned proteins. (B) Cells were starved for 14hours, stimulated with EGF and treated with 100µM Chloroquine for the corresponding times. Samples were harvested, lysed and loaded on 15% SDS-PAGE gels. Western blot membranes were probed with antibodies against the mentioned proteins.

Supplemental Figure 6. HA-LAMTOR3 is found in higher molecular weight bands upon Velcade treatment. SH-LAMTOR3 was tandem affinity purified from HEK293 as described in Material and Methods. An aliquot from the eluates of the first and purification steps were run on SDS PAGE gel, analyzed by Western blotting and the membrane probed with α-ubiquitin antibody. The remaining samples were analyzed on a silver stained gel and the regions corresponding to the α-ubiquitin positive signals were cut, digested and the tryptic digests analyzed using an UltiMate 3000 nano-HPLC system (coupled to an LTQ Orbitrap XL ETD mass spectrometer operating in data dependent mode to switch between MS and MS/MS acquisition. The Proteome Discoverer version 1.3.0.339 with search engine Sequest was used for data analysis. The table provides an overview of the identified LAMTOR3- or ubiquitin-peptides, present in each of the regions analyzed.

Material and Methods of supplemental figures:

In vitro transcription/translation of p21 and p27 - Constructs expressing p21 and p27 under the control of the T7 promoter were used in an *in vitro* transcription/translation reaction using the rabbit reticulocyte system (Promega). Reactions were performed for 90 minutes at 30ºC, according to the Promega kit instructions. Obtained IVTT samples were analyzed by Western blotting to confirm protein production.

Expression and purification of recombinant proteins - Recombinant proteins expressed and purified as described before (1) .

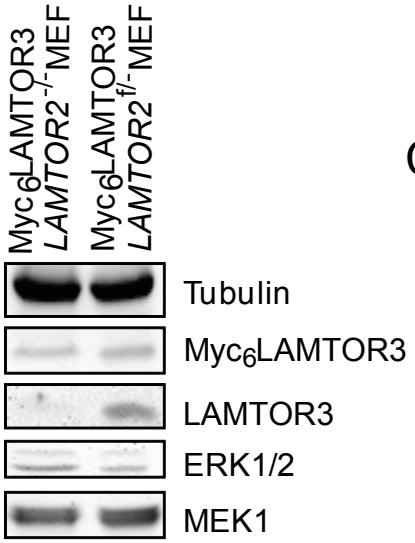
Mass spectrometry analysis of SH-LAMTOR3 tandem affinity precipitation - HEK293 SH-LAMTOR3 were induced with 400ng/ml tetracycline for 24 hours and supplemented or not with 200nM Velcade for the final 7 hours. Tandem-Affinity Purification of SH-LAMTOR3 was performed according to the modified protocol by Gstaiger *et al* (2). The lysates were prepared as described in Material and Methods. Lysates (approximately 200mg of total protein) were loaded on a Biospin columns (Bio-Rad) containing 300µl Strep-Tactin beads (IBA Tagnologies) and gravity drained. The beads were washed with RIPA buffer and subsequently with NP40 buffer (50mM Tris-HCl pH 8, 250mM NaCl, 0.5% NP40, 1mM EDTA, 50mM NaF, 1mM Na_3VO_4, 0.5mM PMSF, 0.5mg/mL pepstatin, 5mg/mL leupeptin, 1mM Pefabloc SC, and the bound protein was eluted in 2.5mM D-biotin in NP40 buffer. One third of the eluate was stored as single step purified SH-LAMTOR3 for subsequent analyses. For the second purification step the biotin eluate from StrepTactin sepharose was added to 200µL α-HA agarose beads (Sigma) and rotated for 1hour at 4°C. The samples were centrifuged and the supernatant removed. The resuspended agarose beads were loaded into a Biospin column (Bio-Rad) and gravity drained. The anti-HA agarose was washed with NP40 buffer and finally with a buffer containing 50mM Tris-HCl pH 8, 250mM NaCl and 1mM EDTA. Retained protein was eluted from the column with 100mM formic acid and immediately neutralised with 1M TEAB buffer (Fluka)(3). TCA-precipitated protein was separated on 15% polyacrylamide gels and either transferred to PVDF membrane for probing with anti-ubiquitin or silver stained (4). Silver gel bands corresponding to α-ubiquitin positive signals in Western blotting, were excised and in-gel digested with trypsin (Promega, Madison, WI, USA) as described by Hellman (5). Tryptic digests were analyzed using an UltiMate 3000 nano-HPLC system (Dionex, Germering, Germany) coupled to an LTQ Orbitrap XL ETD mass spectrometer (ThermoScientific, Bremen, Germany) equipped with a nanospray ionization source. The peptides were separated on a homemade frit-less fused-silica microcapillary column (75 µm i.d. x 280µm o.d. x 10cm length) packed with 3µm reversed-phase C18 material (Reprosil) at a flow rate of 250nL/min. Solvents for HPLC were 0.1% formic acid (solvent A) and 0.1% formic acid in 85% acetonitrile (solvent B). The gradient profile was as follows: 0–2 min, 4% B; 2–55 min, 4–50% B; 55–60 min, 50–100 % B and 60-65 min, 100%B. The LTQ Orbitrap XL ETD mass spectrometer was operating in data dependent mode to switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 250 – 1800) were acquired in the Orbitrap in positive ion mode with a resolution of R = 60,000 (FTMS). MS/MS spectra were obtained in the linear ion trap (LTQ) using collision induced dissociation (CID) and electron transfer dissociation (ETD).

Parameters applied in MS/MS scan events were: minimum signal required 500; isolation width (m/z) 1.0; activation time 30 ms; normalized collision energy 35.0; and activation Q of 0.250; dynamic exclusion was set to 15 sec. MS/MS spectra were acquired in centroid mode with a target value of 1×10^4 respectively 50 ms maximum ionization time. Proteome Discoverer version 1.3.0.339 (ThermoScientific) with search engine Sequest was used for data analysis. Raw data obtained by nano-LC – ESI-MS/MS were searched against the NCBInr Homo sapiens database to which the sequence of SH-tagged protein LAMTOR3 was added. The following settings were applied: Enzyme for protein cleavage was trypsin; two missed cleavages were allowed; precursors mass tolerance was set to 10ppm and the fragment masses tolerance was 0.5Da; false discovery rate (FDR) was set to 0.05 (5%); fixed modification was Carbamidomethylcysteine; oxidation of methionine and GlyGly on lysine were used as variable modifications.

References of supplemental figures:

1. Kurzbauer, R., Teis, D., de Araujo, M. E., Maurer-Stroh, S., Eisenhaber, F., Bourenkov, G. P., Bartunik, H. D., Hekman, M., Rapp, U. R., Huber, L. A., and Clausen, T. (2004) *Proc Natl Acad Sci U S A* **101**, 10984-10989
2. Glatter, T., Wepf, A., Aebersold, R., and Gstaiger, M. (2009) *Molecular systems biology* **5**, 237
3. Rudashevskaya, E. L., Sacco, R., Kratochwill, K., Huber, M. L., Gstaiger, M., Superti-Furga, G., and Bennett, K. L. (2012) *Nature protocols* **8**, 75-97
4. Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis* **8**, 93-99
5. Hellman, U. (2000) *Exs* **88**, 43-54

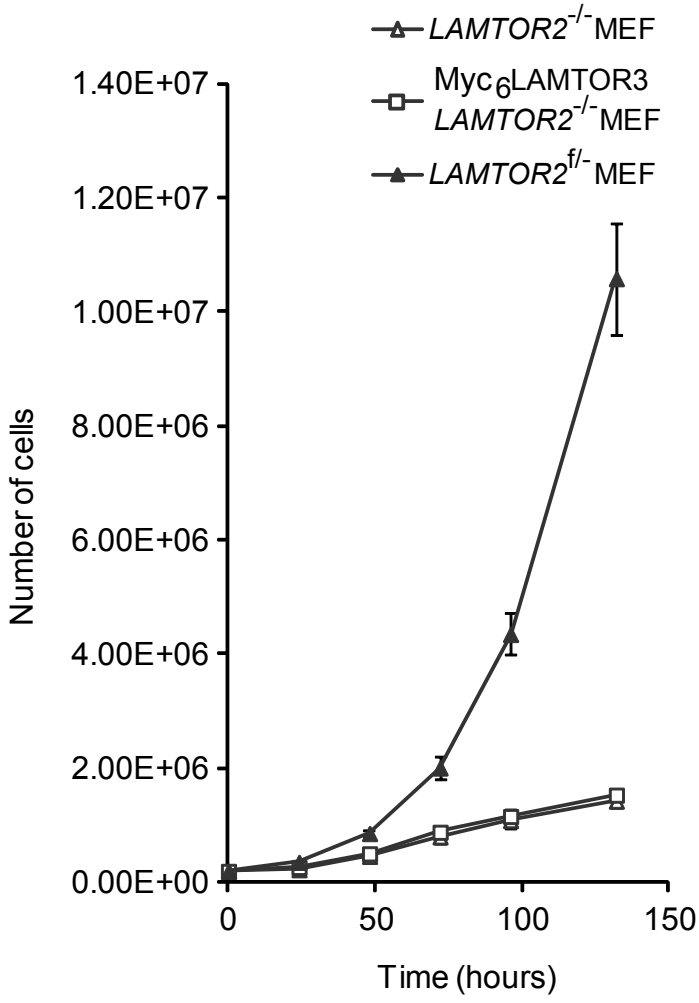
A



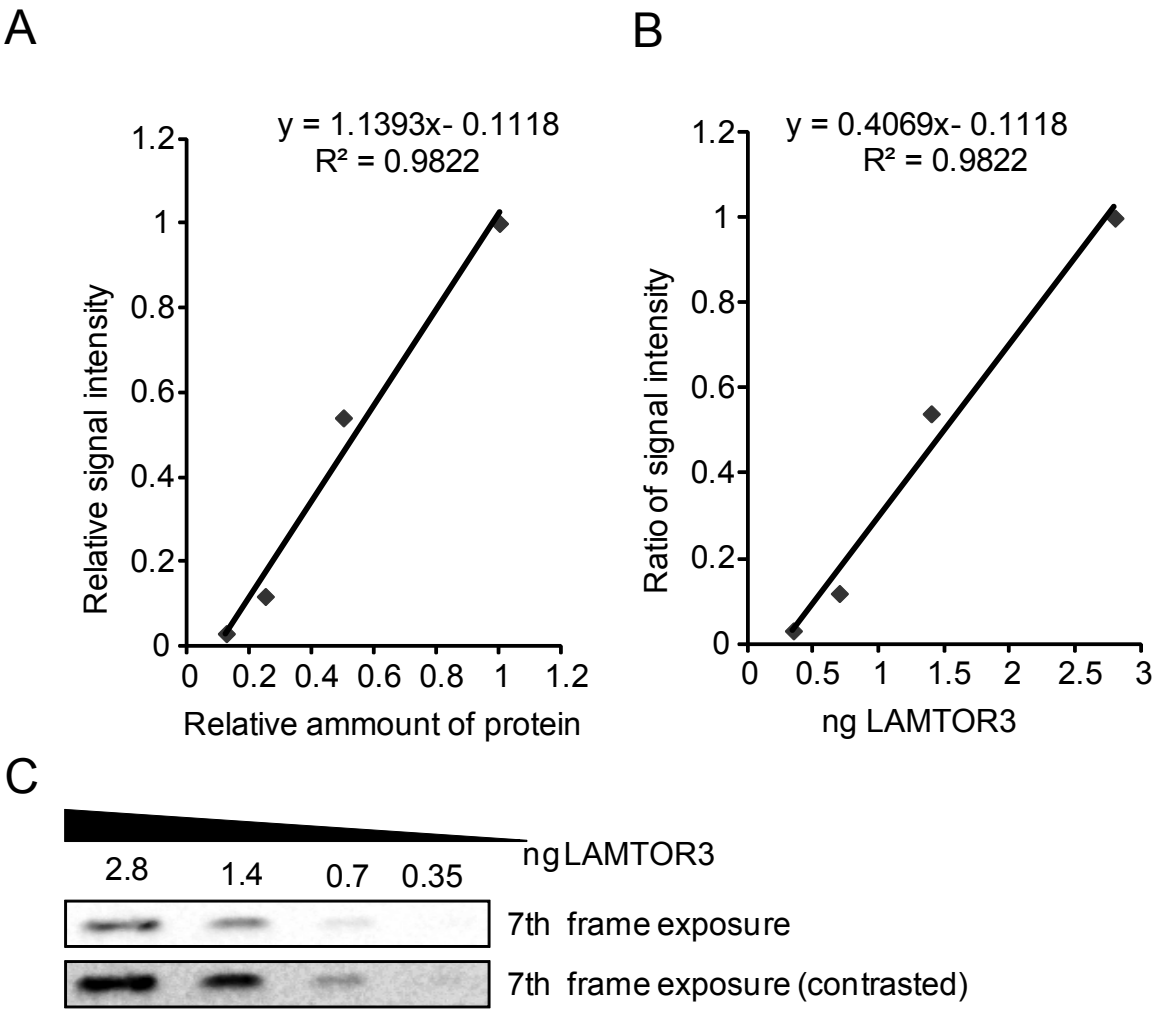
C

*M*ADDLKRFLYKKLPSVEGLHAIVV
SDRDGVPVIKVANDSAPEHALRP
GFLSTFALATDQGSKLGLSKNKSII
C YYNTYQVVQFNRLPLVVSFIAS
SSANTGLIVSLEKELAPLFEELIKV
VEVS

B



Supplemental figure 1.



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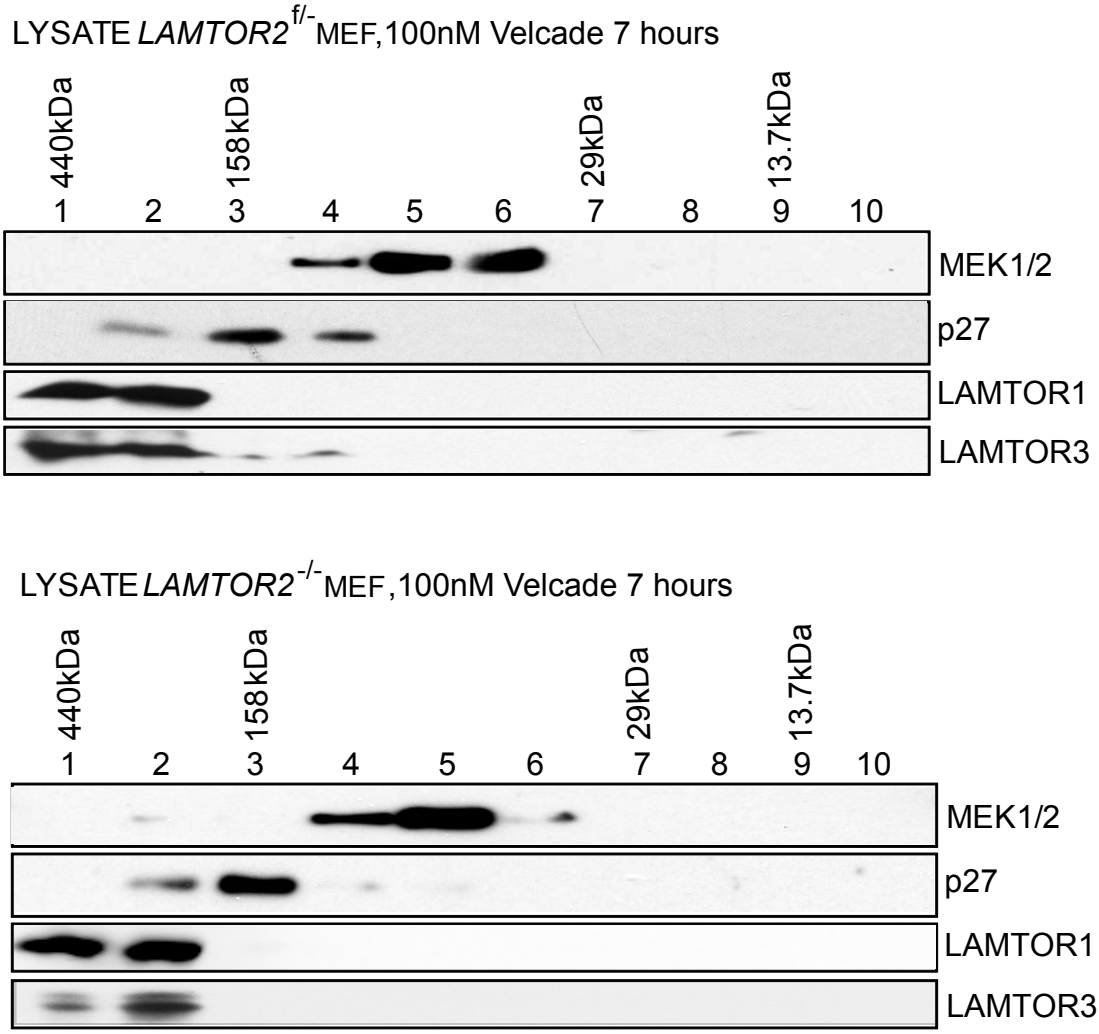
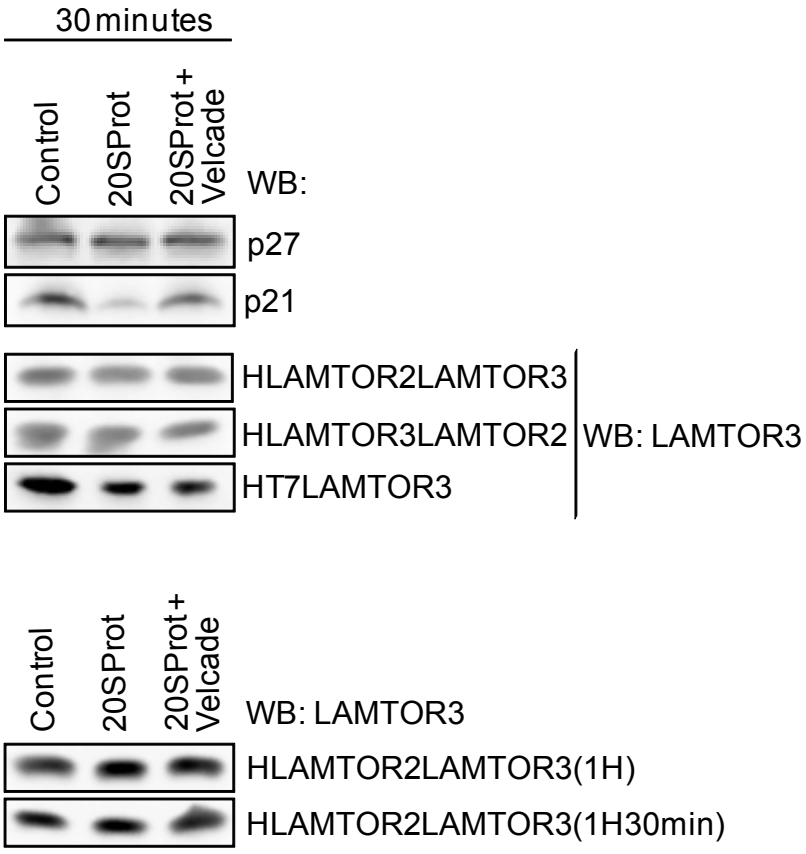


Supplemental figure 3.

A

Alignment of the C terminus of p21 with the N terminus of LAMTOR3

B



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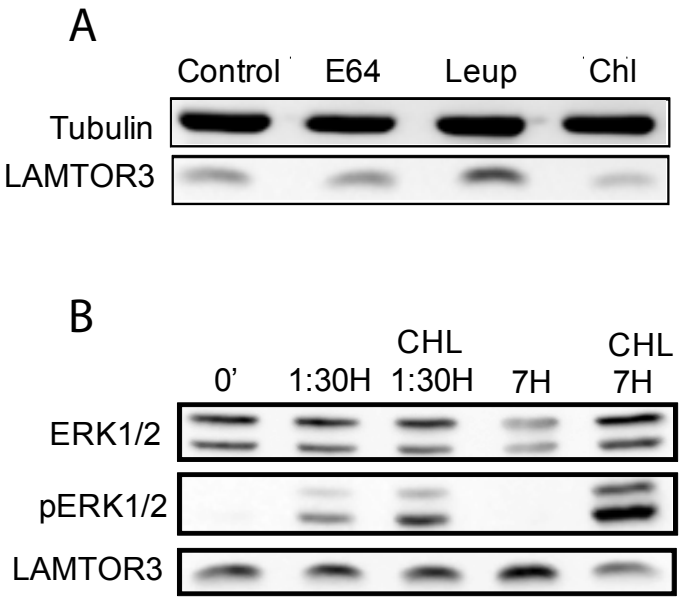
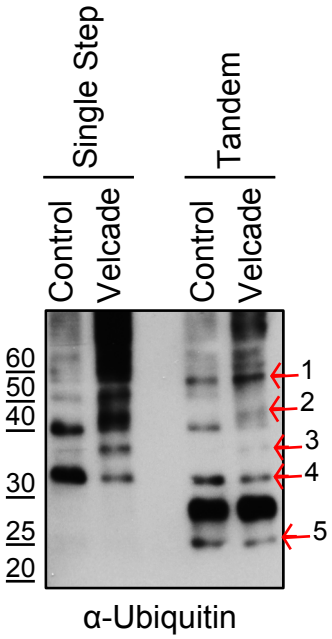


Supplemental figure 5.



Ubiquitin
LAMTOR3

Sample	Aprox. MW	nr. Peptides	Sequence Coverage	Peptide	MH$^+$(Da)	Xcorr CID	Xcorr ETD	Missed Cleavages
1	55	3	44.60%	LIFAGKQLEDGR	1460.79729	3.28	_	1
				ESTLHLVLR	1067.62895	2.51	_	0
				TLSDYNIQK	1081.56023	2.50	_	0
2	44	2	26.10%	VANDSAPEHALRPGFLSTFALATDQGSK	2900.48398	3.91	_	0
				GGGSGGGSGGGSWSHPQFEK	1832.81108	3.34	_	0
				KLPSVEGLHAIVVSDR	1719.97831	_	5.04	0
		2	36.80%	LIFAGKQLEDGR	1460.79766	3.24	2.82	1
				TITLEVEPSDTIENVK	1787.94365	2.73	_	0
3	37	6	43.50%	VANDSAPEHALRPGFLSTFALATDQGSK	2900.46352	4.60	5.36	0
				LPSVEGLHAIVVSDRDGVPVIK	2300.30674	3.93	_	1
				GGGSGGGSGGGSWSHPQFEK	1832.79984	3.76	5.02	0
				KLPSVEGLHAIVVSDRDGVPVIK	2428.40329	3.46	_	2
				AADITSLYK	981.52690	2.41	_	0
				KLPSVEGLHAIVVSDR	1719.97831	_	5.33	1
		3	48.60%	LIFAGKQLEDGR	1460.78899	3.46	_	1
				TITLEVEPSDTIENVK	1787.93376	3.16	_	0
				ESTLHLVLR	1067.62273	2.73	_	0
4	32	9	58.70%	VANDSAPEHALRPGFLSTFALATDQGSK	2900.46645	4.62	5.83	0
				GGGSGGGSGGGSWSHPQFEK	1832.80229	4.06	_	0
				LPSVEGLHAIVVSDRDGVPVIK	2300.30747	3.67	_	1
				KLPSVEGLHAIVVSDRDGVPVIK	2428.40395	3.46	5.67	2
				SIICYYNTYQVVQFNR	2068.01348	2.83	_	0
				ELAPLFEELIK	1301.74028	2.41	_	0
				AADITSLYK	981.52855	2.34	_	0
				LPSVEGLHAIVVSDR	1591.88559	_	4.42	0
				AADITSLYKK	1109.62479	_	3.37	1
		3	49.90%	TITLEVEPSDTIENVK	1787.93840	3.85	_	0
				IQDKEGIPPDQQR	1523.78984	3.39	2.87	1
				ESTLHLVLR	1067.62419	2.37	_	0
5	26	5	43.50%	VANDSAPEHALRPGFLSTFALATDQGSK	2900.47963	4.86	5.45	0
				GGGSGGGSGGGSWSHPQFEK	1832.80974	3.45	_	0
				KLPSVEGLHAIVVSDR	1719.98966	_	4.62	1
				KLPSVEGLHAIVVSDRDGVPVIK	2428.41176	_	4.51	2
				AGSADDLKR	932.48655	_	2.56	1

Supplemental Figure 6