Supporting information

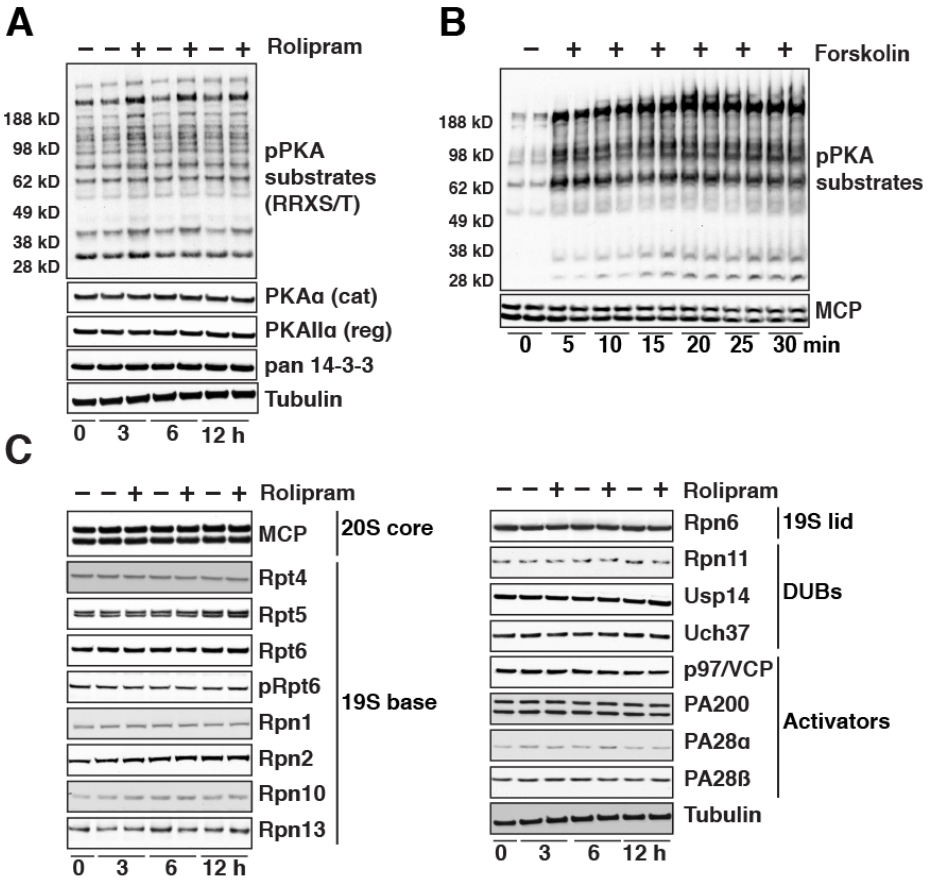

Figure S1 Raising cAMP levels activates PKA but does not increased the levels of proteasome subunits in myotubes when intracellular proteolysis rises.

(A) Inhibition of PDE4 by rolipram enhances the phosphorylation of many cellular proteins**.** Myotubes were treated with or without rolipram and then assayed by immunoblotting for the levels of phosphorylated PKA substrates, catalytic subunit of PKA (PKAα), regulatory subunit of PKA (PKAIIα) and pan 14-3-3. Tubulin was used as loading control. **(B)** Raising cAMP levels with forskolin increases rapidly the phosphorylation of many cellular proteins**.** 293A cells were treated with forskolin for the indicated times, then assayed by Immunoblot for the levels of phosphorylated PKA substrates. MCP was used as loading control. **(C)** Inhibition of PDE4 by rolipram does not affect the levels of 26S proteasome subunits in myotubes. Myotubes were treated as in (A), followed by immunoblot for MCP (20S, α -subunits, α1, 2, 3, 5, 6 & 7), 19S base (Rpt4, Rpt5, Rpt6, pRpt6, Rpn1, Rpn2, Rpn10 and Rpn13) 19S lid (Rpn6), Deubiquitinating enzymes (DUBs) (Rpn11, Usp14 and Uch37), and proteasome activator (p97/VCP, PA200, PA28α and PA28β) in total lysates. Tubulin was used as loading control.

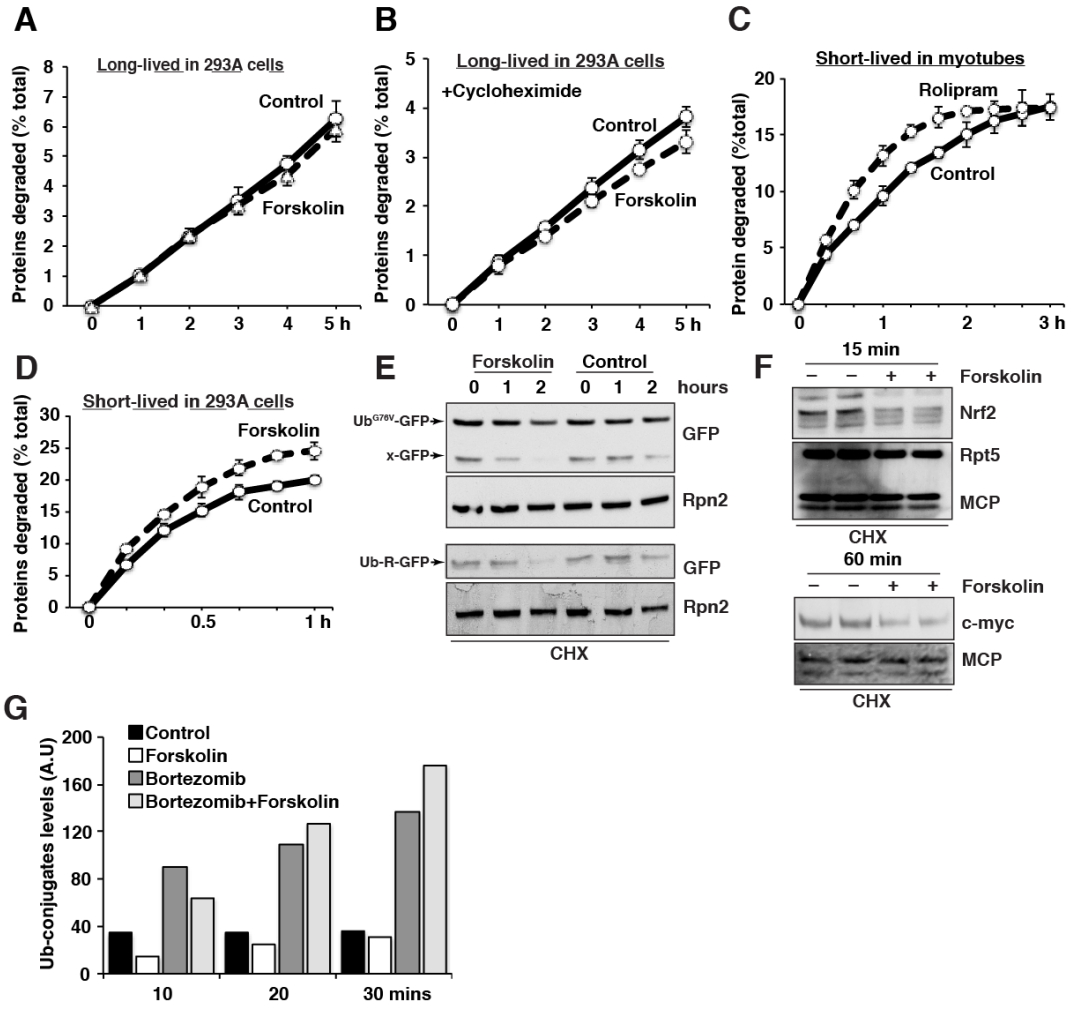

Figure S2 Raising cAMP levels promotes the degradation of short- but not long-lived proteins as well as model substrates of the UPS.

(A) and **(B)** Raising cAMP levels with forskolin does not affect the degradation of long-lived cell proteins. 293A cells were incubated with [^{3}H]-tyrosine for 24 h and then washed with chase medium for 2 h. New chase media containing non-radioactive tyrosine (2 mM), DMSO, forskolin (50 μM) or CHX (100 mg/ml) were added, and media samples were collected as indicated. The released radioactivity was measured and plotted as a percentage of the total radioactivity in proteins at 0-time. Error bars represent SEM. n=4. **(C)** and **(D)** Raising cAMP levels with forskolin promotes the degradation of short-lived proteins. To follow degradation of short-lived and long-lived proteins differentiated myotubes were pre-treated with or without rolipram for 3 h **(C)** or 293A cells **(D)** incubated with [^{3}H]tyrosine for 10 min to label both short-lived and long-lived components and then washed with chase medium 3 times. Myotubes **(C)** or 293A **(D)** cells were treated with media containing non-radioactive tyrosine (2 mM), and either rolipram, forskolin, or nothing. Media samples were collected as indicated. The released radioactivity was measured as in **(A)**. Error bars represent SEM. n=4. Because long-components were not degraded faster (Fig. S2A and S2B), the increase must be due to

breakdown of short-lived proteins. Because no change was seen upon labeling long-lived proteins selectively, this increase must be due to breakdown of short-lived proteins. **(E)** Forskolin treatments enhance the degradation of model substrates of UPS, N-end rule substrate and UFD pathway substrate. 293A cells were transfected with Ub-R-GFP (N-end rule substrate), and Ub^{G76V}-GFP (UFD pathway substrate) were treated with or without forskolin in the presence of CHX to prevent the protein synthesis and levels of GFP was analyzed by immunoblotting. Rpn2 was used as loading controls. **(F)** Raising cAMP levels with forskolin promotes the degradation of endogenous short-lived proteins in 293A cells. 293A cells were treated with forskolin for the indicated time and the levels of Nrf2 and c-myc were analyzed by immunobloting. Rpt5 and MCP were used as loading controls. **(G)** Forskolin-induced rapid reduction of Ub conjugates was prevented by treatment of Bortezomib. Levels of ubiquitinated proteins levels were estimated from immunoblot of Figure-1J by densitometry and plotted against treatment time.

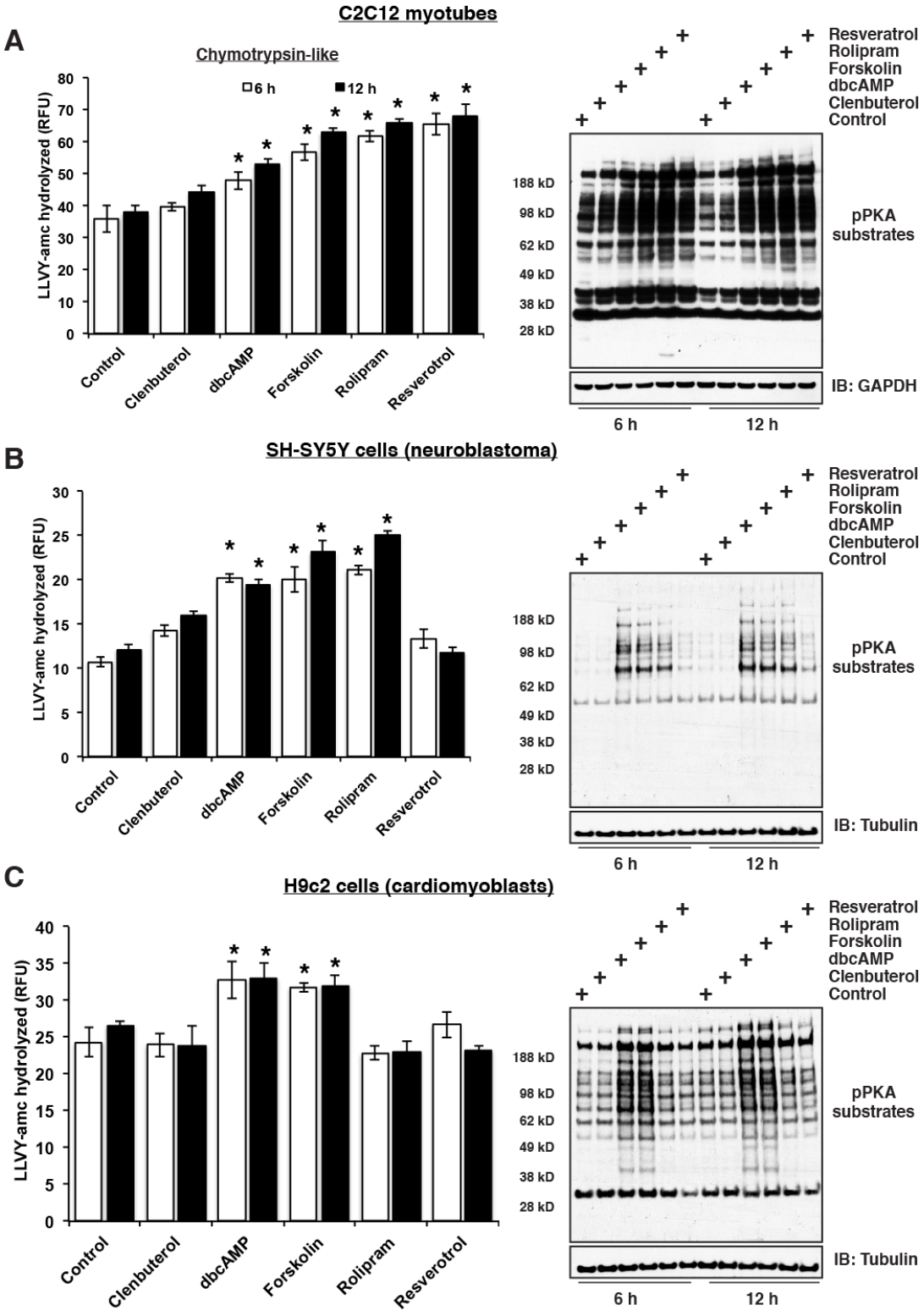

Figure S3 Activation of PKA enhances the phosphorylation of many cellular proteins and enhances peptide hydrolysis by proteasomes in myotubes, neuroblastoma and cardiomyoblasts cells.

(A), (B) and **(C)** Differentiated myotubes **(A)**, SH-SY5Y neuroblastoma cells **(B)** or H9c2 cardiomyoblasts **(C)** were treated with DMSO, clenbuterol (10 μM), dbcAMP (10 μM), forskolin (50 μM), rolipram (50 μM), or resveratrol (10 μM) as indicated, and chymotrypsin-like peptidase activity was measured in cell extracts by following hydrolysis of suc-LLVY-amc (Left) and represented by relative fluorescence units (RFU). Error bars represent SEM. n=4, *p<0.01. Levels of phosphorylated PKA substrates in cell extracts were assayed by immunoblot (Right). Tubulin was used as loading control.

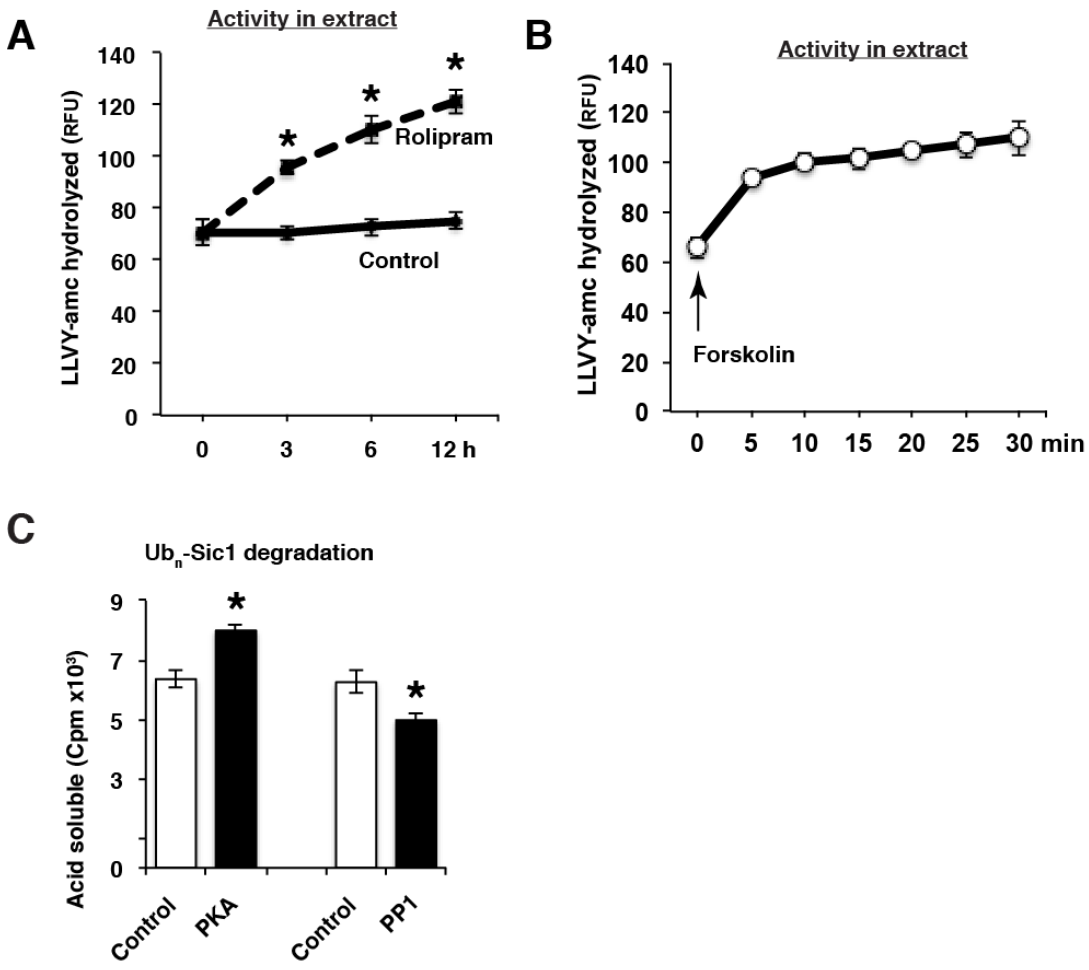

Figure S4 Activation of adenylate cyclase with forskolin stimulates proteasomal activity rapidly while inhibition of PDE4 with Rolipram stimulates proteasomal activity more slowly.

(A) Differentiated myotubes were treated with or without rolipram, as indicated, and chymotrypsin-like peptidase activity was measured in cell extracts using suc-LLVY-amc and represented by relative fluorescence units (RFU). Error bars represent SEM. n=6, *p<0.01. **(B)** 293A cells were treated with DMSO or forskolin for the indicated times. Chymotrypsin-like peptidase activity was measured in cell extracts using suc-LLVY-amc and represented by relative fluorescence units (RFU). Error bars represent SEM. n=6. **(C)** *In vitro* treatment of 26S proteasomes from control myotubes with PKA promotes the degradation of ubiquitinated Sic1 and PP1 treatment reduces it. Purified 26S proteasomes from control myotubes treated with PKA or PP1 for 90 min at 30°C. Ub$_n$-Sic1 degradation was measured after 10 min incubation. Error bars represent SEM. n=3, *p<0.01.

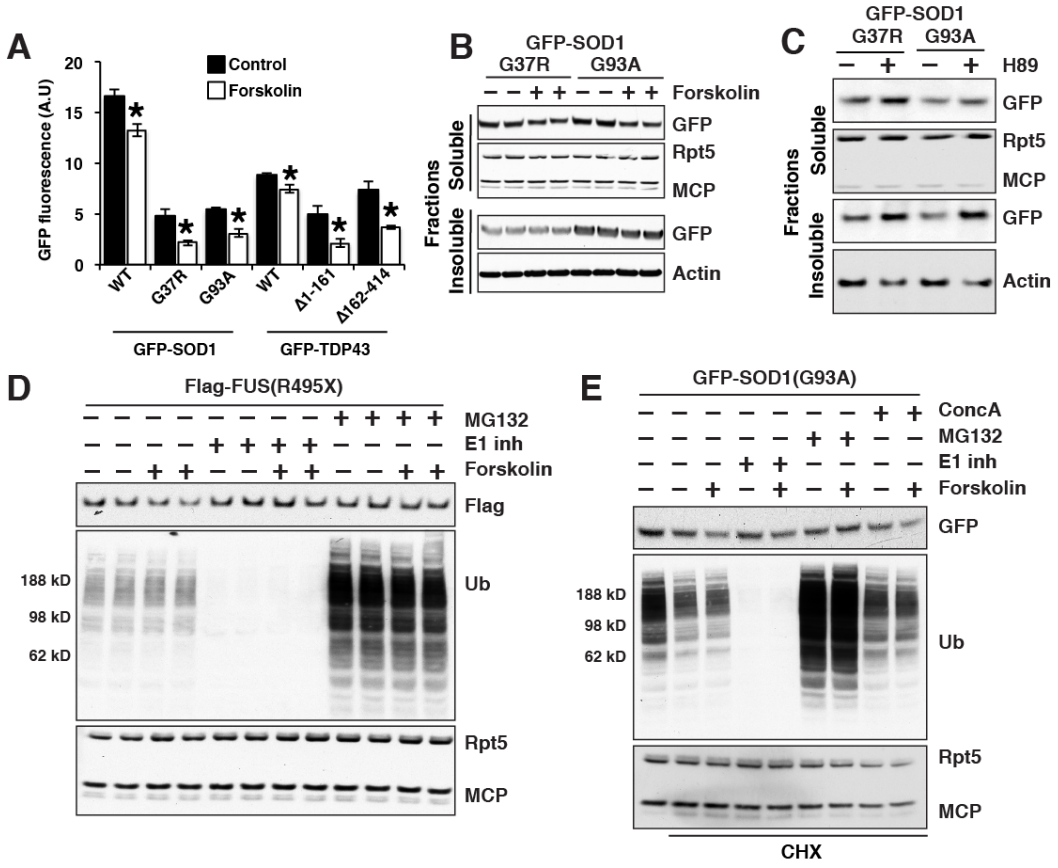

Figure S5 Raising cAMP with forskolin enhances the degradation and PKA inhibitor H89 causes accumulation of aggregation-prone proteins.

(A) Forskolin enhances degradation of GFP fusion proteins associated with neurodegenerative diseases. 293A cells transfected with GFP-SOD1-WT, GFP-SOD1-G37R, GFP-G93A, GFP-TDP43-WT, GFP-TDP43-Δ1-161 and GFP-TDP43-Δ162-414. After 48h transfection cells were treated with vehicle (DMSO) or forskolin for 5 h in the presence of CHX. GFP fluorescence was measured in live cells at 488/509 nm. Error bars represent SEM. n=8, *p<0.01. **(B)** and **(C)** Raising cAMP with forskolin enhances the degradation and PKA inhibitor H89 causes accumulation of SOD1 mutant proteins. 293A cells were transfected with SOD1 (G37R and G93A). After 48 h of transfection, cells were treated with control or Forskolin **(B)** or H89 **(C)** for 5 h in the presence of CHX. Immunoblot analysis was performed both in 1% Triton-X 100 soluble and insoluble fractions against GFP (SOD1). Levels of Rpt5 and MCP (soluble fraction), Actin (Insoluble fraction) were used as loading control. **(D)** and **(E)** Forskolin caused a rapid decrease in the levels of aggregation-prone proteins by the UPS not autophagy. 293A cells were transfected with Flag-FUS(R495X) GFP-SOD1(G37R) or GFP-SOD1(G93A). After 48 h of transfection, cells were treated with DMSO, forskolin, E1 inhibitor (1 μM), MG132 (10 μM), ConcA (0.2 μM), or combinations thereof, for 5 h in the presence of CHX. Levels of Flag (FUS-R495X) **(D)** or GFP (SOD1-G93A) **(F)** were analyzed by immunoblot. Rpt5 and MCP were used as loading controls.

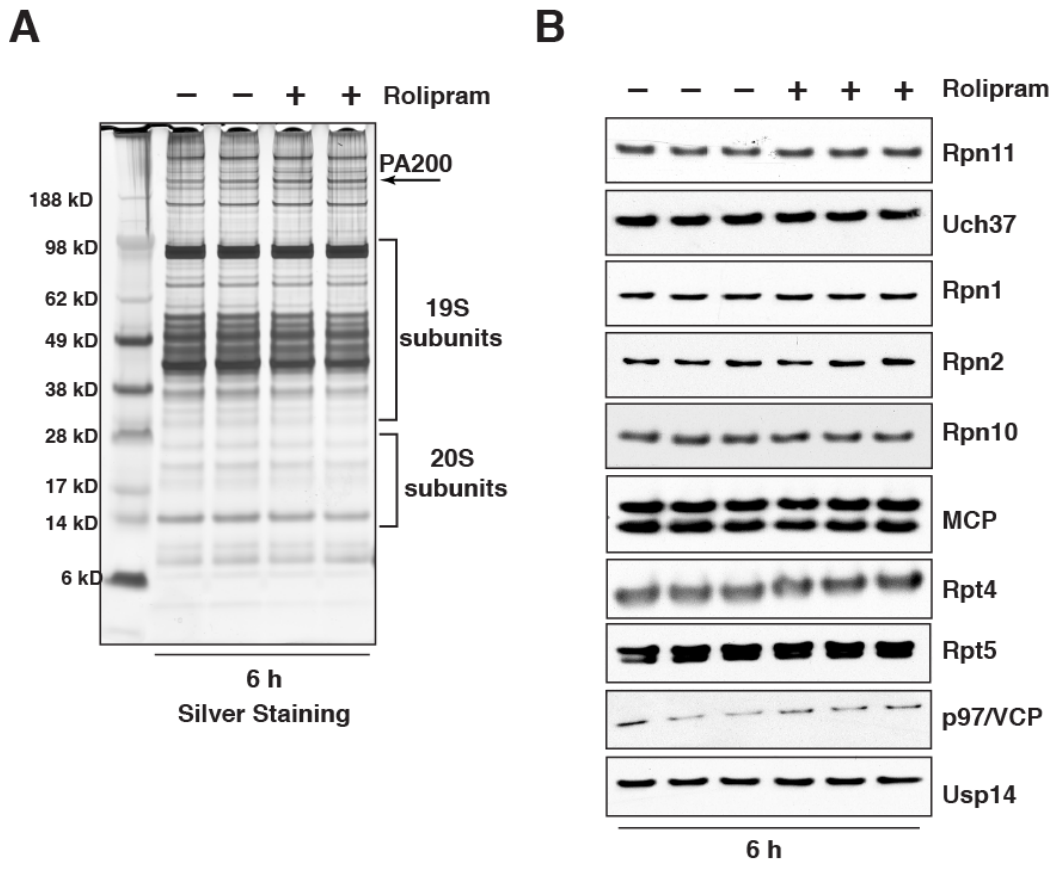

Figure S6 Treatment of myotubes with rolipram does not cause clear change in the levels of subunits purified 26S proteasomes except perhaps PA200.

(A) No apparent difference was evident in subunit composition of proteasomes purified by the UBL method except at molecular weight approximately to PA200, an increase in proteasome activator PA200/BLM10 was also confirmed by western blotting (Fig-5A) and quantitative mass spectrometry (Supplemental table-1). Myotubes were treated with vehicle or rolipram for 6 h, proteasomes purified and analyzed by SDS-PAGE followed by silver staining. The arrow represents the PA200. **(B)** Equal amount purified 26S proteasomes from myotubes treated with or without rolipram for 6 h was subjected to immunoblot analysis of Rpn11, Uch37, Rpn1, Rpn2, Rpn10, Rpn13, MCP, Rpt4, Rpt5, p97/VCP and USP14.

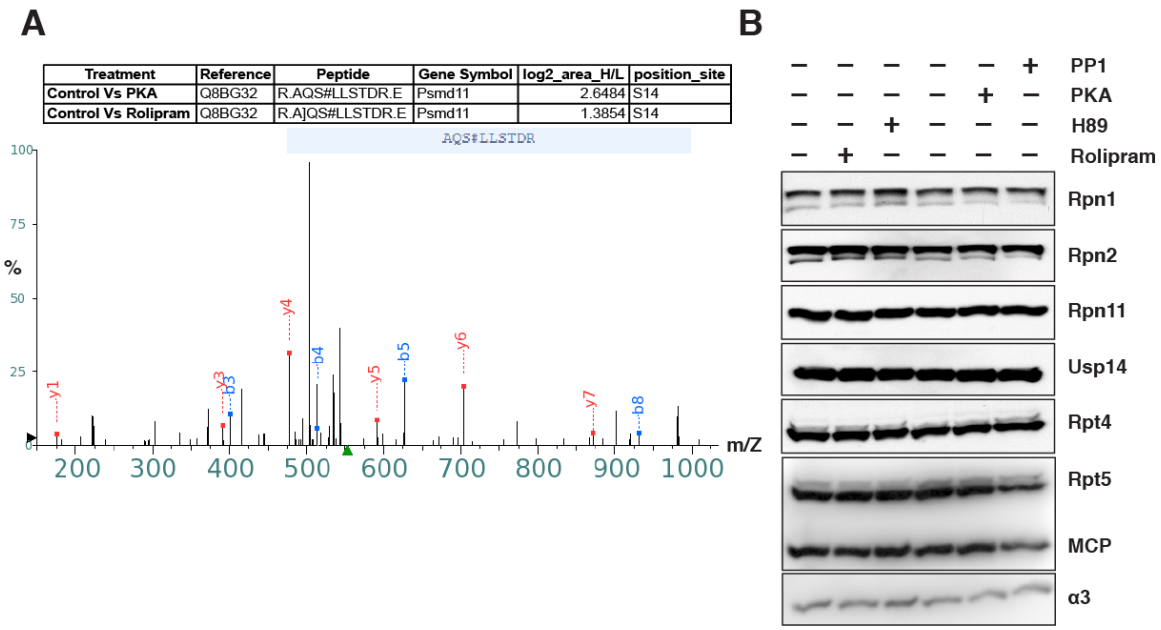

Figure-S7 Treatment of myotubes with rolipram enhances the phosphorylation of 19S subunit, Rpn6 but not other proteasome subunits.

(A) Phosphorylation of Rpn6 at serine 14 was identified by phosphoproteomics. The mass spectrometry spectrum obtained after phosphorylation of Rpn6 from the purified proteasomes isolated from myotubes treated with rolipram for 6 h. **(B)** Treatment of myotubes with rolipram does not cause phosphorylation other proteasome subunits. Equal amounts of purified 26S proteasomes from myotubes treated with or without rolipram for 6 h and 26S proteasomes from control myotubes were treated with PKA or PP1 for 90 min at 30°C was subjected Zn^{2+}-Phos-tag SDS-PAGE followed by immunoblot analysis of Rpn1, Rpn2, Rpn11, Usp14, Rpt4, Rpt5, MCP and, α3.

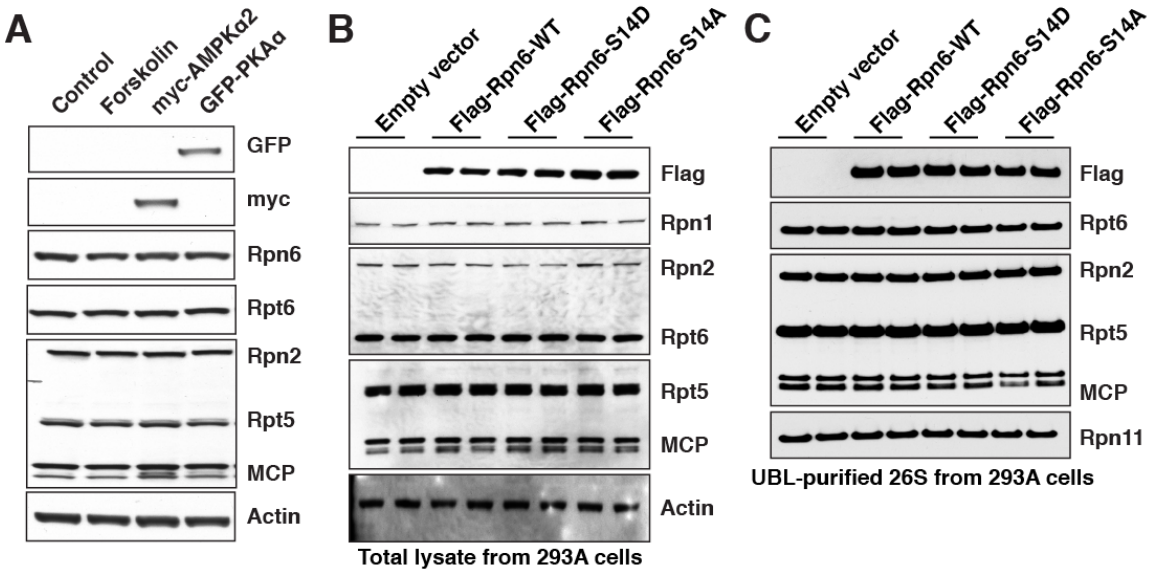

Figure-S8 Overexpression of kinases (myc-AMPKα or GFP-PKAα) or Rpn6 mutants (Flag-Rpn6-WT, -S14D or –S14A) does not affect the levels of proteasome subunits

(A) 293A cells were transfected with control empty vector, myc-AMPKα or GFP-PKAα. Control transfected cells were treated with control or Forskolin for 5 h and was subjected to immunoblot analysis of GFP, myc, Rpn6, Rpt6, Rpn2, Rpt5, MCP and Actin. **(B)** 293A cells were transfected with control empty vector, Flag-Rpn6-WT, -S14D and -S14A and was subjected to immunoblot analysis of Flag, Rpn1, Rpn2, Rpt6, Rpt5, MCP and Actin. **(C)** 293A cells were transfected with empty vector control, Flag-Rpn6-WT, -S14D or –S14A. After 48 h post-transfection, 26S proteasomes purified by UBL-method and subjected to immunoblot analysis for Flag, Rpt6, Rpn2, Rpt5, MCP and Rpn11.

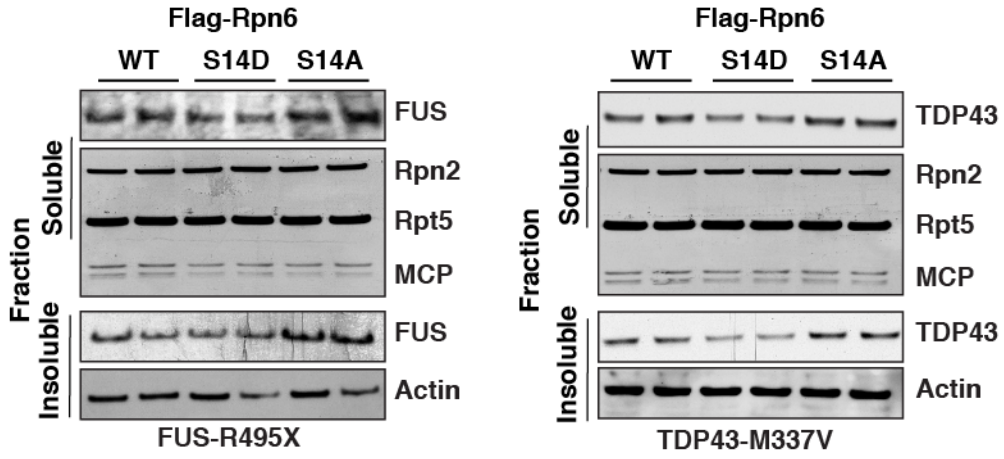

Figure S9 Overexpression of Rpn6-S14D mutant decreased the levels of aggregation-prone proteins in both soluble and insoluble fractions.

Co-transfection of FUS-R495X or TDP43-M337V with Flag-Rpn6-WT, -S14D or –S14A into 293A cells. Immunoblot analysis was performed both in 1% Triton-X 100 soluble and insoluble fractions against FUS and TDP43. Levels of Rpn2, Rpt5 and MCP in the soluble, Actin in the insoluble fraction were used as loading control.

Materials and methods

Reagents and plasmids

Forskolin (#F6886), rolipram (#R6520), clenbuterol (#C5423), cycloheximide (#C7698) and dibutyryl-cAMP (dbcAMP) (#D0627) were purchased from Sigma, USA. Resveratrol (#554325) was from Calbiochem, USA, MG132 (#1748) from R&D Biosystems, USA, concanamycin A (#202111A) from Santa Cruz Biotechnology, USA, H89 (#BML-EI196) from Enzo Life Sciences, USA, and glutathione sepharose 4B from GE Healthcare Life sciences, USA. cAMP-dependent protein kinase (PKA) (#P6000S), Protein phosphatase 1 (PP1) (#P0754S), and Protein phosphatase inhibitor 2 (I-2) (#P0755S) were purchased from NEB, USA. Ub_5-DHFR, Ube1 inhibitor (ML00603997) and bortezomib (Velcade) were kindly provided by Millennium pharmaceuticals, USA. [^{3}H]-tyrosine (#NET127005MC) was purchased from Perkin Elmer, USA, and phenylalanine, L-[3,4,5-^{3}H] (#ART 0614) from American Radiolabeled Chemicals, USA. Information pertaining to the plasmids and their resources is given in table below.

Construct name	Provider/Reference
Flag-FUS(WT)	Kindly provided by Dr. Robin Reed, Harvard Medical School, Boston, MA, USA (1)
Flag-FUS(R495X)	
Flag-TDP43(WT)	
Flag-TDP43(M337V)	
Flag-TDP43(G298S)	
TDP43(WT)	Kindly provided by Proteostasis Therapeutics, Cambridge, MA, USA
TDP43(M337V)	
Tau(WT)	
Tau(P301L)	
SOD1(WT)	
SOD1(G93A)	
GFP-CL1	Kindly provided by Dr. Ron Kopito, Stanford University, Stanford, CA, USA(2)
Ub-M-GFP	Obtained from Addgene (#11938) (3)
Ub-R-GFP	Obtained from Addgene (#11939) (3)
Ub-^{G76V}-GFP	Obtained from Addgene (#11941) (3)

pAMPK α2 WT	Obtained from Addgene (#15991) (4)
pcDNA3-mouse PKA-cα-mEGFP	Obtained from Addgene (#45521) (5)
GFP-SOD1(WT)	Kindly provided by Dr. Piera Pasinelli, Farber Institute for Neurosciences, Thomas Jefferson University, Philadelphia, PA, USA
GFP-SOD1(G93A)	
GFP-SOD1(G37R)	
GFP-TDP43(WT)	
GFP-TDP43(Δ1-161)	
GFP-TDP43(Δ162-414)	
Flag-Rpn6-WT	Purchased from Origene company
pQTEV-PSMD11	Obtained from Addgene (#31333) (6)
Flag-Rpn6-S14D	Cloned by using the following primers with QuikChange II Site-Directed Mutagenesis Kit FP:CGGTCGGTGCTGAGTAGGTCCTGGGCT CTCTGGAACT RP:AGTTCCAGAGAGCCCAGGACCTACTCAG CACCGACCG-3'
Flag-Rpn6-S14A	Cloned by using the following primers with QuikChange II Site-Directed Mutagenesis Kit FP:GTCGGTGCTGAGTAGGGCCTGGGCTCT CTGGAA RP:TTCCAGAGAGCCCAGGCCCTACTCAGCA CCGAC

Cell culture and treatments

C2C12 myoblasts (ATCC #CRL-1772), 293A cells (ThermoFisher Scientific, #R705-07), H9c2 (rat cardiomyocytes) (ATCC #CRL-1446), SH-SY5Y neuroblastoma cells (ATCC #CRL-2266) were grown in DMEM containing 10% fetal bovine serum and 100 U/ml penicillin and streptomycin. Differentiated C2C12 myotubes (7), 293A cells, H9c2 (rat cardiomyocytes), SH-SY5Y cell were treated with control, rolipram (50 μM), dbcAMP (10 μM), resveratrol (10 μM), clenbuterol (10 μM), or forskolin (50 μM) for the times indicated in Results.

Measurement of protein degradation

Protein degradation rates were assayed as described elsewhere (7). To measure the rate of long-lived protein degradation, cells were labeled for 24 h with 5 µCi/ml of L-[3,5-^{3}H]tyrosine or L-[3,4,5-^{3}H]phenylalanine. Cells were washed 3 times with 1× PBS and incubated for further 2 h with a large excess of non-radioactive tyrosine or phenylalanine (2 mM) to avoid radiolabel reincorporation. Cells were then washed, and media replaced with fresh chase media containing DMSO, rolipram or forskolin in addition to non-radioactive tyrosine or phenylalanine. Aliquots of the media were taken at various chase times. To measure rates of short-lived protein degradation, labeling time was reduced to 10 min, and chase was started immediately after washing.

Proteins in the media aliquots were precipitated with TCA, pelleted by centrifugation at 20,800×*g* for 15 min at 4°C, and TCA-soluble radioactivity was measured. For each time point, cells were solubilized in 0.2 N NaOH, and radioactivity was measured. Total radioactivity is the sum of this residual radioactivity in cells and the TCA-soluble radioactivity at different time points. Protein breakdown was expressed as [^{3}H]tyrosine or [^{3}H]phenylalanine released over time as a percentage of total incorporated [^{3}H]tyrosine or [^{3}H]phenylalanine.

Cells transfections

Transfection of 293A cells was performed using Lipofectamine 2000 transfection reagent (Life Technologies, USA) by following the manufacturer's instructions. Treatments were started after 48 h of transfection.

Live GFP reading

After 48 h of transfection cells were treated with indicated drugs with indicated time and the GFP fluorescence was measured in live cells at 395/509 nm.

Cell lysis and Immunoblotting (IB)

Cells were washed with cold PBS and harvested by scraping. After pelleting at 1000×*g*, 4°C, 2 min, cells were resuspended in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, Complete protease inhibitor cocktail (Roche, USA), 2 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1× Phosphatase inhibitor cocktail (Thermo Scientific, USA), gently rocked for 15 min at 4°C and centrifuged at 20,800×*g* for 15 min at 4°C. Total protein content was quantified using Coomassie Plus staining (Thermo

scientific, USA). Lysate amounts were standardized to total protein content and subjected to SDS-PAGE using 4–12% Bis-Tris pre-cast gels (Life Sciences, USA). Proteins were transferred to nitrocellulose membranes. Immunoblotting was performed using the antibodies and their resources were given in below table.

Antibodies name	Catalog number	Company name/Provider
anti-pPKA (RRXS/T)	9624	Cell signaling technology
anti-pPKA (RXXS/T)	9621	Cell signaling technology
anti-Ubiquitin (P4D1)	SC-8017	Santa Cruz biotechnology
anti-Tubulin	T9026	Sigma
anti-PKA alpha (cat)	SC-903	Santa Cruz biotechnology
anti-PKAII alpha (reg)	SC-909	Santa Cruz biotechnology
anti-Pan 14-3-3	SC-629	Santa Cruz biotechnology
anti-MCP	BML-PW8195	Enzo Life Sciences
anti-Rpt4/PSMC6	BML-PW8830	Enzo Life Sciences
anti-Rpt5/PSMC3	BML-PW8770	Enzo Life Sciences
anti-Rpt6/PSMC5	BML-PW9265	Enzo Life Sciences
anti-pRpt6		Gift from Dr. Gentry Patrick (8)
anti-Rpn1/PSMD2	SC-68352	Santa Cruz biotechnology
anti-Rpn2/PSMD1	SC-166038	Santa Cruz biotechnology
anti-Rpn10/PSMD4	BML-PW9250	Enzo Life Sciences
anti-Rpn13/ADRM1	AB21749	Abcam
anti-Rpn6/PSMD11	NBP1-46192	Novus Biologicals
anti-Rpn11/PSMD14	4197	Cell signaling technology
anti-Usp14	SC-100630	Santa Cruz biotechnology
anti-Uch37	3904	Epitomics
anti-P97/VCP	2649	Cell signaling technology
anti-PA200	PA1-1961	Thermo scientific
anti-PA28 alpha	2408	Cell signaling technology
anti-PA28 beta	2409	Cell signaling technology
anti-Flag	M2	Sigma
anti-FUS		Gift from Dr. Robin Reed (1)
anti-TDP43	3449	Cell signaling technology

anti-Tau	A0024	Dako
anti-pTau (pS396/404)		Gift from Dr. Dr. Peter Davies
anti-Alpha-synuclein	SC-7011-R	Santa Cruz biotechnology
anti-Gapdh	G8795	Sigma
anti-Nrf2	SC-722	Santa Cruz biotechnology
anti-c-myc	SC-40	Santa Cruz biotechnology
anti-HDAC1	GTX100513	GeneTex
RNA pol II	05-623-Z	Millipore
anti-pSer/Thr	PP2551	ECM Biosciences
anti-Sarcomere (MF20)	MF20	DSHB
anti-Actin	A2543	Sigma
anti-GFP	GFP-12A6	DSHB
anti-rabbit HRP conjugate	W4011	Promega
anti-mouse HRP conjugate	W4021	Promega

Affinity purification of 26S proteasomes

Differentiated myotubes treated with DMSO (control) or rolipram were collected from ten 150-mm dishes (Total 30, 3 biological replicates and 10 each) to purify 26S proteasomes using the ubiquitin-like domain (Ubl) method described previously with slight modifications (9, 10). Cells were harvested by scraping in cold PBS and centrifuged. The cell pellet was resuspended in buffer containing 25 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 150 mM NaCl and Phosphatase inhibitor cocktail (#88667 Thermo Scientific, USA) and lysed by sonication (5 bursts of 10 seconds each with 50 seconds rest on ice). The crude lysate was centrifuged at 3,000×*g*, 4°C, for 5 minutes. The supernatant was centrifuged for 1 h at 100,000×*g*, 4°C. The resulting clarified lysates (100,000×*g*) were incubated for 2 h at 4°C with 2 mg purified GST-Ubl protein (9, 10) and a corresponding amount of glutathione-sepharose. After 2 h of incubation, the slurry containing 26S proteasomes bound to GST-sepharose was poured into and empty disposable column and washed with buffer. Proteasomes were eluted by incubating the resin with excess purified His-UIM (2 mg/ml) at 4°C for 15 min (9, 10). Elution was repeated, and eluates combined. His-UIM was removed from the eluate by incubating with Ni^{2+}-NTA-agarose for 20 min at 4°C. Concentration of 26S proteasomes was calculated based on total protein content and assuming a molecular weight of 2.5 MDa.

Measuring peptidase activity of purified 26S proteasomes

Proteasome peptidase activity have been measured using a protocol described elsewhere (9, 10). Briefly, the activity of proteasomes after affinity purification of 26S (~1 nM) or in extract (1-2 µg) was measured with 20 µM of fluorogenic substrates (suc-LLVY-amc, suc-LLE-amc and suc-VLR-amc) in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT. Fluorogenic hydrolysis was monitored at λ_{ex}=380 nm and λ_{em}=460 nm at 37°C for 60 min. Rate of hydrolysis was calculated based on the slope of fluorescence increase over time between 20-60 min (linear phase of the reaction).

Measuring peptidase activity of 26S proteasomes in total cell extract

Peptidase activity of 26S proteasome in total cell extract was performed as described elsewhere (11). Briefly, the assay was performed in 100 µl reaction containing 1 µg of total cell protein extract, 50 mM Tris-HCl pH8.0, 10 mM MgCl2, 1 mM ATP, 1 mM DTT, 25 µM Suc-LLVY-AMC. Fluorescence was measured as described above.

Degradation of ubiquitinated proteins by purified 26S proteasomes

Ub_5-DHFR (dihydrofolate reductase) provided by Millennium Pharmaceuticals (Takeda oncology company) has radiolabeled using PKA and [γ-^{32}P]ATP as described previously (12-14). Sic1 was radiolabeled with [^{35}S]ATPγS using casein kinase II (NEB). Sic1 ubiquitination was carried out using Rsp5, Ubc4, E1 and free ubiquitin, as described previously (12-14). Degradation of substrates (~50 nM) by purified 26S proteasomes (~1 nM) was assayed in a buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.01 mg/ml BSA (Sigma) as reported before (12-14) and measured by following the conversion of substrates to TCA-soluble radiolabeled peptides (12-14).

ATPase assay of purified 26S proteasomes

ATP hydrolysis experiments were performed as described before (12-14). Briefly, ATP hydrolysis by 26S proteasomes was measured using the malachite green assay that detects the release of free phosphate after hydrolysis of ATP. ATPase activity and hydrolysis by proteasomes (5–10 nM) were measured in a buffer containing 25 mM Hepes/KOH, pH 8, 2.5 mM $MgCl_2$, 125 mM potassium acetate, 0.025% Triton X-100, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA as reported previously (13-15). Stimulation of

ATPases was measured by including a 100-fold molar excess of casein and linear hexaubiquitin at equimolar concentrations.

Treatment of 26S proteasomes with Protein kinase A and Protein phosphatase 1

Approximately 50nM of purified 26S proteasomes were incubated with pure Protein kinase A (PKA) (NEB #P6000S) or Protein phosphatase 1 (PP1) (NEB, #P0754S) for 60-90 min at 30^{O}C and then measure the 26S proteasomal activities as described above.

Cell fractionation

Cell fractionation was performed by lysing in 1% Triton-X buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% [v/v] Triton X-100, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM Na3VO4, 0.1 mM NaF, and a cocktail of protease inhibitors by mixing for 20 min at 4^{O}C. Total lysate was centrifuged at 12,500*xg* for 30 min. The supernatant were used as Triton-X-soluble fraction. The pellet was resuspended in a buffer containing 50 mM Tris-HCl (pH 7.4) plus 2% SDS, sonicated briefly (10 seconds, twice), and analyzed as Triton-X-insoluble fractions.

MS/MS analysis of affinity purified 26S proteasomes

Quantitative mass spectrometry was performed on Ubl affinity purified 26S proteasomes from myotubes treated with either control (DMSO) or rolipram for 6 hours. Briefly, 26S proteasomes were subjected quantitative mass spectrometry and phospho proteomics analysis as described elsewhere (16).

Zn^{2+}-Phos-tag SDS-PAGE

Detection of phosphorylated in by western blotting was performed using Zn^{2+}-Phos-tag SDS gels (17) (Wako, Japan) by following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using Student's *t*-tests, one-way post-hoc Tukey HSD. All values are expressed as means ± SEM. Asterisks represent *p*-values, *p≤0.01.

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