Supporting Information for

The SUMOylation landscape of renal cortical collecting duct cells

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Supporting Excel files provided separately

DETAILED METHODS

Cell culture, transfection and antibodies. Mouse kidney cortical collecting duct cells (mpkCCD14) were cultured as described[1]. Cells were stably transfected using standard protocols for Lipofectamine 2000 (Invitrogen), with pEFIRESpuro vectors containing N-terminal hexa-histidine tagged SUMO1 (6xHis-SUMO1^{T95K}) and SUMO2 (6xHis-SUMO2^{T90K})[2]. For mouse Pept2 (Slc15a2) studies, mpkCCD14 cells were transfected with codon optimized flag-tagged mouse Pept2 cloned into a pcDNA3.1(+)-N-DYK vector (GenScript, sequence available on request). Stably expressing cells were selected using 2 µg/ml puromycin (Sigma-Aldrich) and named mpkCCD-SUMO1^{T95K} or mpkCCD-SUMO2^{T90K} cells. For Pept2 transfected cells 0.15 µg/ml Geneticin (cat# 10131-019, ThermoFischer) was used. Antibodies utilized were against SUMO1 (cat# AM1200a, Abgent), SUMO2 (cat# AM1224a, Abgent), 6 x His (cat# A00186, GenScript), FLAG-tag (cat# F7425, Sigma-Aldrich), Proteasome 20s (cat# sc67339, Santa Cruz) and α-actin (cat# A2066, Sigma-Aldrich).

Cell experiments. mpkCCD-SUMO1^{T95K} or mpkCCD-SUMO2^{T90K} cells were cultured as described[3] in 175 cm^2 flasks until fully confluent and switched to pure DMEM/F-12 GlutaMAX the day before experiments. In general, four T175 flasks were required to yield 60-90 mg of crude protein lysates for affinity purification. For heat shock studies, cells were subjected to 1 h at 43°C in a standard incubator. For other experiments, cells were treated with 10 µM MG132 (Sigma-Aldrich) for five hours, with the last hour also containing 22 µM PR619 (Abcam). SILAC experiments were performed as described[3] with cells cultured until confluent in 2 x T175 cm^2 flasks, then switched to experimental media (standard media with addition of HEPES, D-Glucose and respectively light or heavy amino acids) for 48 h. Cells were treated for 24 h with either 1 µM aldosterone (light labelled cells) or DMSO vehicle (heavy labelled cells).

Immunoblotting. Standard procedures were utilized and western blots were developed using ECL detection. Images were acquired with ImageQuant LAS 4000 (GE Healthcare). Non-saturated images were used for quantification by determining signal intensity in each band using Image Studio-Lite 5.2 (LI-COR Biosciences). Data was analyzed using an unpaired students t-test with GraphPad Prism 7. A p-value < 0.05 was considered significant.

Real-time quantitative polymerase chain reaction (RTqPCR). Cells transfected with mouse Pept2 were treated with aldosterone or DMSO as described above. RNA extraction and RTqPCR were performed as previously described [4]. Primer pairs utilized were: forward 5'– ACA CAG GAA TCA AAC CAG CCA–3', reverse 5'–CCA TAG TCT TTG CCC ACG CT–3'. Data was analyzed using an unpaired students t-test using GraphPad Prism 7. A *p-value* < 0.05 was considered significant.

Immunocytochemistry and confocal laser scanning microscopy. Cells were cultured on glass coverslips until confluent. For experiments with aldosterone, experiments were similar to as described above. Immunolabeling was performed as previously described[5] using 6x His tag antibody (at 1:5000, cat# MA1-135, ThermoFischer), SUMO1 (at 1:100, cat# AM1200a, Abgent) and SUMO2 (at 1:250, cat# AM1224a, Abgent) antibodies.

Affinity purification with Ni^{2+}-coated agarose beads. Cells were lysed in lysis buffer (8 M urea, 2 M thio-urea, 100 mM sodium phosphate (pH 8), 10 mM Tris (pH 8), 10 mM imidazole, 0.1% SDS, 1% HALT Protease inhibitor (ThermoFischer) and 22 µM PR619). Lysates were sonicated on ice and centrifuged at 71,500 x g for 1 h at 25°C. Protein concentrations of the supernatants were determined by Bradford assay, before 2.5 mM of the reducing agent β-Mercaptoethanol (B-ME) was added to the lysate. Approximately 60 mg of protein lysate was mixed with 800 µl HisPur™ Ni-NTA Resin (ThermoFischer) in a 10 ml spin column overnight (maximum 18 h) at 4°C with rotation. Beads were washed three times in wash buffer 1 (8 M Urea, 2 M Thio-Urea, 100

mM Sodium Phosphate (pH 8), 10 mM Tris (pH 8), 10 mM Imidazole and 2.5 mM B-ME), followed by two washes in wash buffer 2 (8 M Urea, 2 M Thio-Urea, 100 mM Sodium Phosphate (pH 6.3), 10 mM Tris (pH 8), 10 mM Imidazole and 2.5 mM B-ME) and finally two washes in wash buffer 1. Proteins were eluted using successive 20 minute incubations with 1 ml elution buffer (8 M Urea, 2 M Thio-Urea, 100 mM Sodium Phosphate (pH 6.3), 10 mM Tris (pH 8) and 400 mM Imidazole). Elutions were combined before subsequent processing.

Filter-aided sample preparation (FASP). Ni-NTA purified proteins were loaded onto Vivacon 500, 30kD cut-off spin columns (Sartorius), and washed three times with 500 µl UA buffer (8 M Urea and 100 mM Tris (pH 8)). Proteins were incubated for one hour at 56°C with 50 mM DTT in UA buffer followed by incubation in the dark for 20 minutes at room temperature with 50 mM 2-chloroacetamide in UA buffer. Following centrifugation, filters were washed twice with 300 µl IAP buffer (50 mM MOPS-NaOH (pH 7.2), 10 mM Na_2HPO_4 and 50 mM NaCl) and proteins digested overnight at 37°C with 1:50 lysyl endopeptidase (Wako Pure Chemical Corporation) in IAP buffer. Peptides were collected by centrifugation (Lys-C digested peptides) and the remaining larger peptides on the filters were subjected to digestion with 1:100 glycyl endopeptidase (ThermoFischer) at 25°C overnight (Lys-C + Glu-C digested peptides).

K-GG immunoprecipitation (KGG-IP). Peptides were purified using a KGG-IP kit (Cell Signaling Technology). Briefly, 3 µl of packed resin were added to an estimated 250 µg peptides, the beads washed three times with 200 µl IAP buffer and then samples incubated overnight at 4°C with end-over-end mixing. Beads were collected by centrifugation and washed four times with 300 µl ice-cold IAP buffer. Finally, the antibody purified peptides were extracted two times using 10 minutes incubations in 150 µl of 0.15% (v/v) TFA. The eluate was vacuum dried before LC-MS/MS analysis. In one batch of cells, Lys-C peptides were subjected to high-pH peptide

fractionation using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher) according to the manufacturer's instructions, with the modification of collecting only 4 fractions, at 7.5%, 12.5%, 17.5% and 50% ACN respectively. These separate fractions were subjected to K-GG immunoprecipitation.

Nano-liquid chromatography and tandem mass spectrometry (LC-MS/MS) and data analysis. Purified peptides were subjected to LC-MS/MS analysis. Q-Exactive settings (ultra-sensitive) were a loop count of 1, and a maximum injection time of 1000 ms. To ensure high accuracy spectra, the resolution of the precursor scan was set to 70,000 while that of the fragment scan was set to 35,000. HCD collision energy was set at 30 %. Dynamic exclusion was set at 30 s, and precursor ions with charge state unknown, +1 and above +8 were excluded for fragmentation. Sites profiling data was analyzed by MaxQuant (version 1.5.5.1). All the raw files from one SUMO family (SUMO1 or SUMO2) were searched together against the mouse Uniprot database (10^{th} of October 2017), with Lys-C/P as enzyme for group 0 (Lys-C digested peptides) and LysC/P plus GluC for group 1 (Lys-C, followed by GluC digested peptides). Acetylation of protein N-term, oxidation of methionine, phosphorylation of serine, threonine and tyrosine, and di-glycine modification of lysine were set as variable modifications, while carbamidomethylation of cysteine was set as a fixed modification. All other parameters were default.

MS data analysis for SILAC experiment. Data were searched with Proteome Discoverer (v2.1.0.81) with both Sequest and Mascot (v2.5). Two parallel processing nodes, corresponding to LysC and LysC+GluC digest were set up. Settings were highly similar to MaxQuant search, except the SILAC related parameters as follows: in both processing nodes, except for the aforementioned variable modifications, heavy version of di-glycine-modified Lys was manually set in both Sequest and Mascot. Results from these two parallel processing nodes were

transferred to a consensus node, where quantification was done with the default precursor quantification parameters.

Bioinformatics. *Motif analysis***.** A 31 amino acid-long sequence, with the SUMOylated lysine central, was generated for every identified SUMO site by MaxQuant. All such sequences generated for both SUMO1 and SUMO2 were combined, the redundancy was removed and they were analyzed by SequenceLogo (https://www.phosphosite.org/sequenceLogoAction). *G-protein coupled receptor (GPCR), kinase, transcription factor (TF), E3 ligase, deubiquitinating enzyme (DUB), transporter and channel analysis.* The identified SUMO proteins from mpkCCD14 cells and ubiquitylated proteins from mouse kidney[6] were cross-referenced with online databases, to categorize proteins. Databases used were: GPCR-Ligand Association Database[7] (https://zhanglab.ccmb.med.umich.edu/GLASS/); Mouse KinBase (http://kinase.com/web/current/kinbase/genes/SpeciesID/10090/fasta/protein/); Animal Transcription Factor DataBase (TFDB) 2.0[8] (http://bioinfo.life.hust.edu.cn/AnimalTFDB2/); Ubiquitin and Ubiquitin-like Conjugation Database[9] (UUCD, http://uucd.biocuckoo.org/); transporter and channel[10] (https://hpcwebapps.cit.nih.gov/ESBL/Database/NephronRNAseq/Transporters_and_Channels.html). All databases used were also provided in **Table S1**. *STRING analysis.* Protein-protein interactions between SUMOylated transcription factors (TFs) and known water and electrolyte transporters/channels[10] were assessed using STRING[11] (version 10, https://string-db.org/). Organism was set as *Mus musculus* while all other parameters were left as default. Interactions with at least medium confidence (interaction score ≥ 0.4) between a TF and a transporter were retained (**Table S4**), while interactions with highest confidence (interaction score ≥ 0.7) are shown in **Figure S2**. *Clue GO analysis.* SUMO1 and SUMO2 unique and shared proteins (with ≥ 2 SUMO sites in both SUMO1 and SUMO2) were assessed using Clue GO (**Figure 4A, B and C**).

Ingenuity Pathway Analysis (IPA). SUMO1 and SUMO2 unique proteins were assessed individually using core analysis with default parameters. The two core analysis results were subjected to comparison analysis to generate heatmaps (**Figure 4D and E**). For diseases and biofunctions analysis a log10 p value cut-off was set at 10, while for canonical pathway analysis the cut-off was set at 2. Both employed hierarchical clustering as the sorting method.

Data Availability. The mass spectrometry proteomics data for SUMO profiling have been deposited to the ProteomeXchange Consortium via the PRIDE[12] partner repository with the dataset identifier PXD013346, and the SILAC data with the dataset identifier PXD013363.

Supplemental References for Methods

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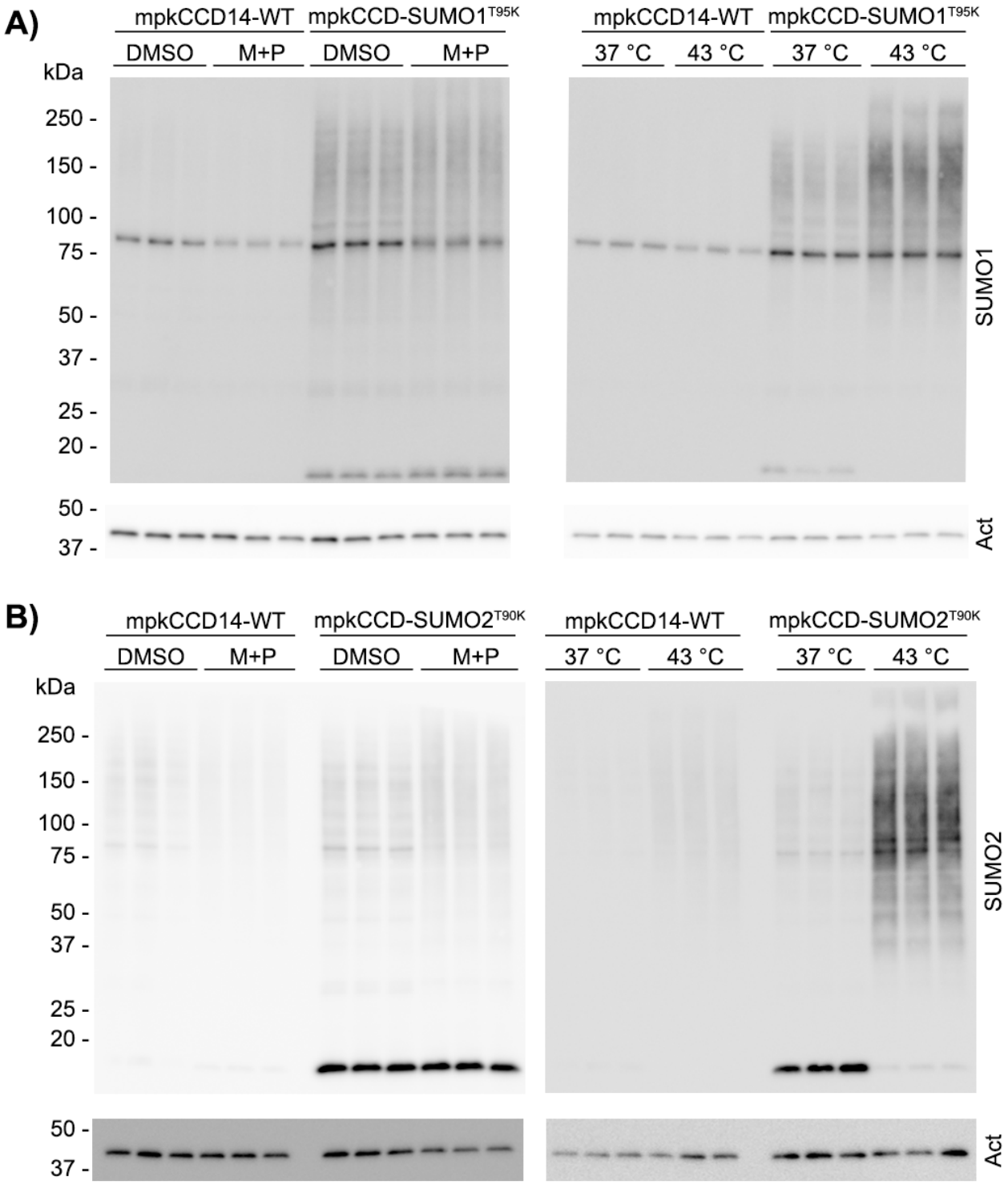


Figure S1. Comparison of SUMOylation levels following various treatments that may enhance SUMOylation. A) Lysates from mpkCCD14-WT and mpkCCD-SUMO1^{T95K} cells under control conditions (37°C) or after heat shock (43°C), vehicle (Veh) or MG132 + PR619 treatment. B) Similar experiments in mpkCCD14-WT/mpkCCD-SUMO2^{T90K} cells. Antibodies used were against SUMO1, SUMO2, and α-actin (Act).

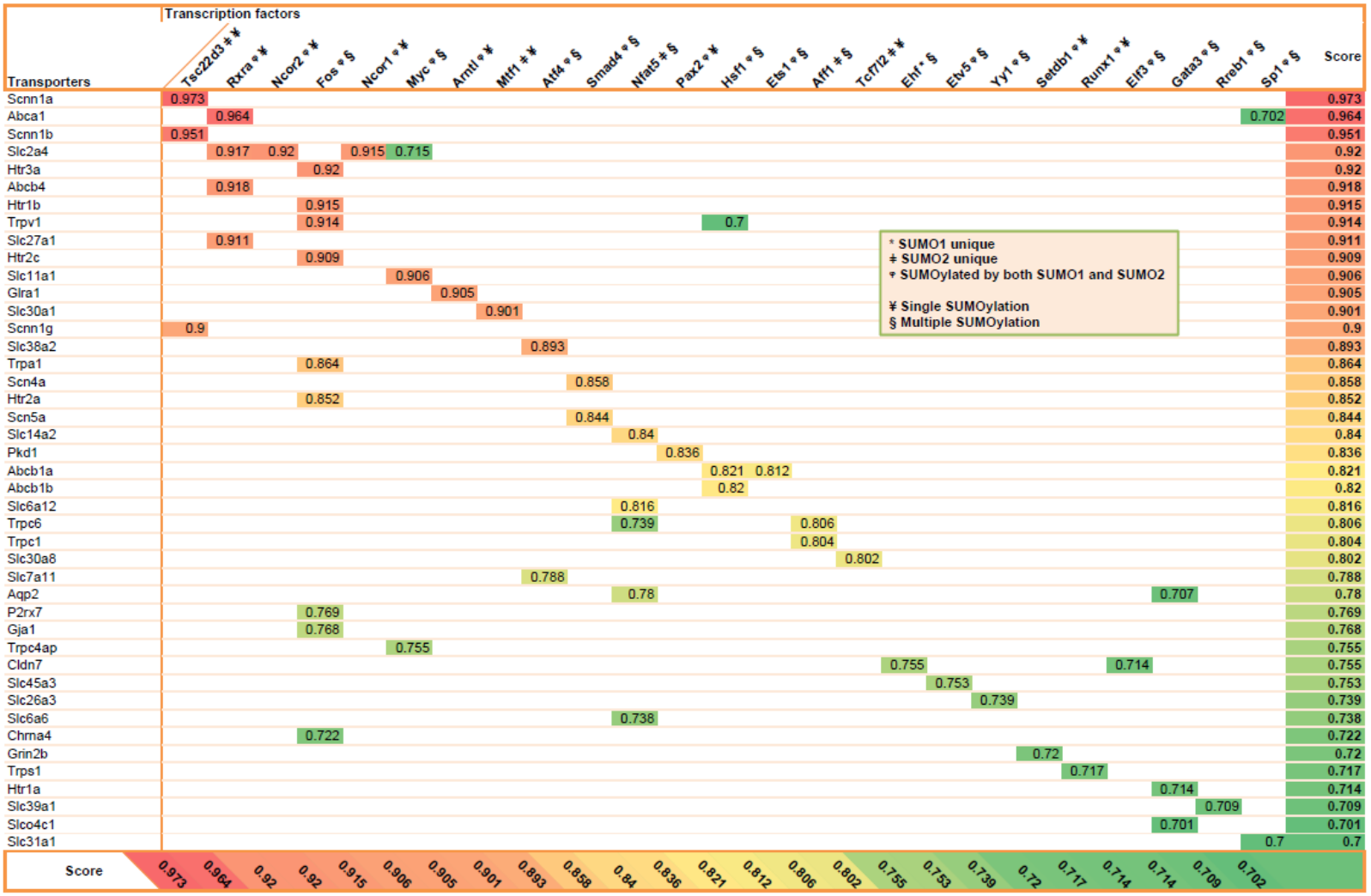


Figure S2. STRING interaction scores. STRING interactions with a score above 0.7 (high confidence) between SUMOylated transcription factors and known renal transporters/channels.

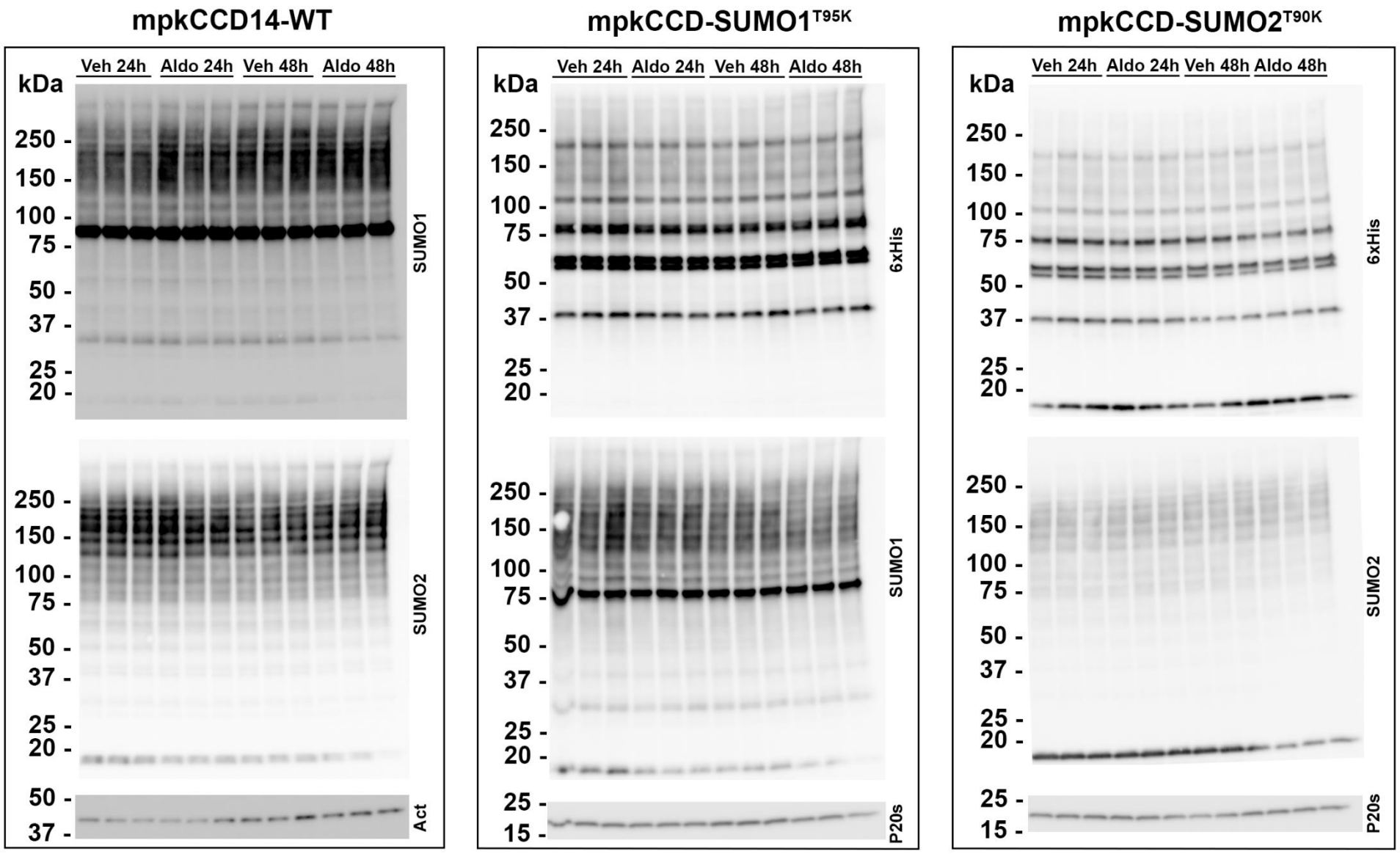
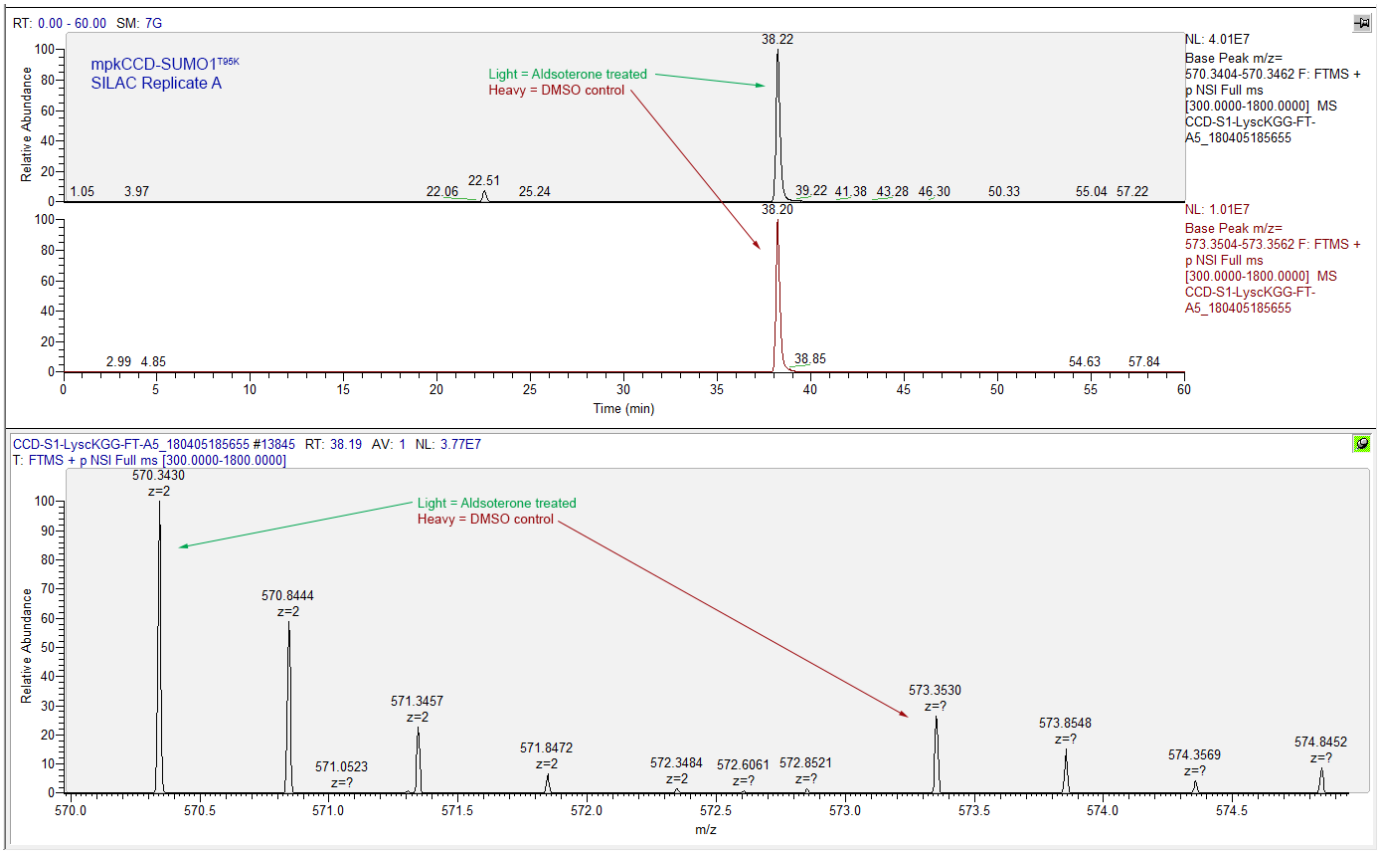


Figure S3. Immunoblotting for SUMO following 24 h and 48 h aldosterone (1 µM) treatment. mpkCCD14-WT, mpkCCD-SUMO1^{T95K} and mpkCCD-SUMO2^{T90K}, in time matched control (Veh) and aldosterone (Aldo) treatment.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SILAC Replicate A
Light = Aldsoterone treated
Heavy = DMSO control
Relative Abundance
Time (min)
NL: 4.01E7
Base Peak m/z= 570.3404-570.3462 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S1-LyscKGG-FT-A5_180405185655
NL: 1.01E7
Base Peak m/z= 573.3504-573.3562 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S1-LyscKGG-FT-A5_180405185655
38.22
38.20
38.85
22.51
CCD-S1-LyscKGG-FT-A5_180405185655 #13845 RT: 38.19 AV: 1 NL: 3.77E7
T: FTMS + p NSI Full ms [300.0000-1800.0000]
570.3430 z=2
570.8444 z=2
571.3457 z=2
571.8472 z=2
571.0523 z=?
572.3484 z=2
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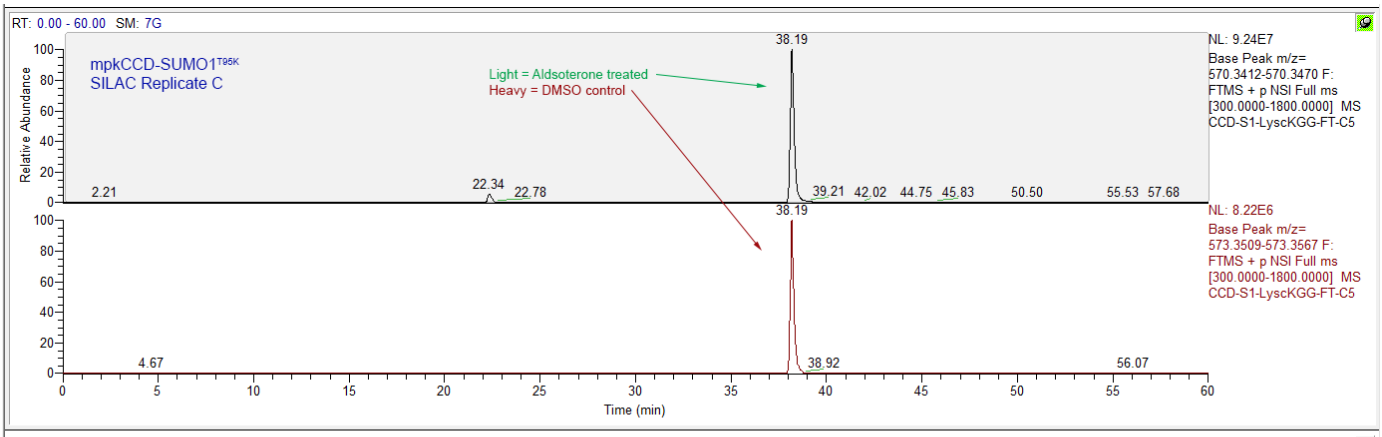
m/z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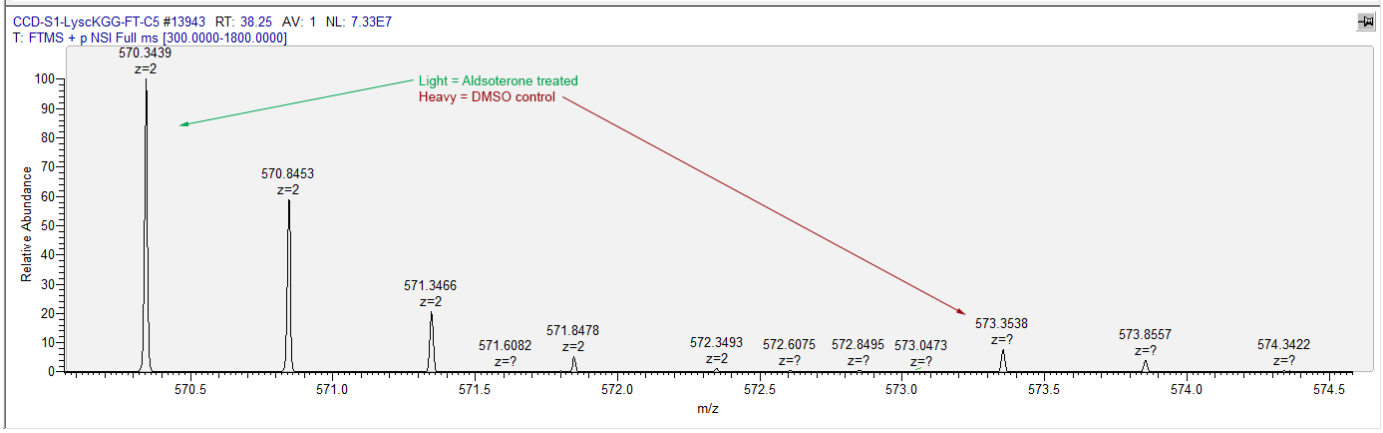
SILAC Replicate C
Light = Aldsoterone treated
Heavy = DMSO control
38.19
NL: 9.24E7
Base Peak m/z= 570.3412-570.3470 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S1-LyscKGG-FT-C5
NL: 8.22E6
Base Peak m/z= 573.3509-573.3567 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S1-LyscKGG-FT-C5
Relative Abundance
Time (min)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Light = Aldsoterone treated
Heavy = DMSO control
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570.8453 z=2
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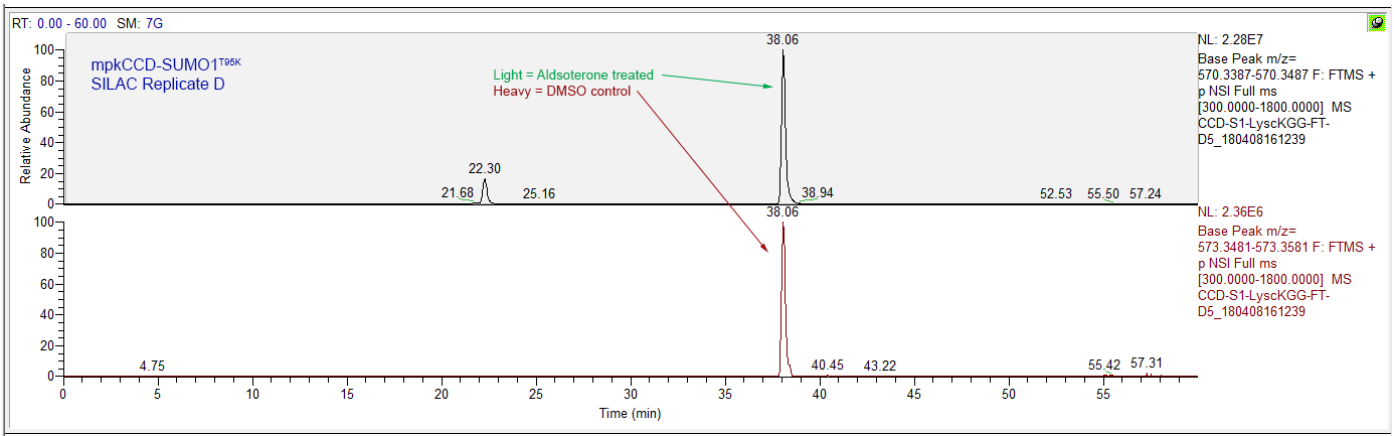
571.8478 z=2
573.3538 z=?
Relative Abundance
m/z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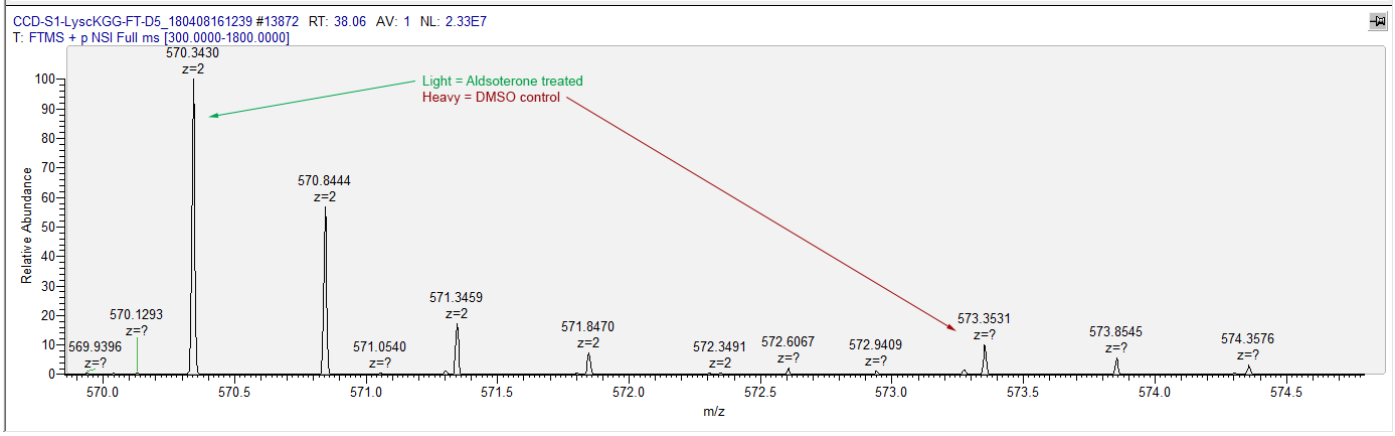
mpkCCD-SUMO1T95K
SILAC Replicate D
Light = Aldsoterone treated
Heavy = DMSO control
38.06
NL: 2.28E7
Base Peak m/z= 570.3387-570.3487 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S1-LyscKGG-FT-D5_180408161239
NL: 2.36E6
Base Peak m/z= 573.3481-573.3581 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S1-LyscKGG-FT-D5_180408161239
Relative Abundance
Time (min)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Light = Aldsoterone treated
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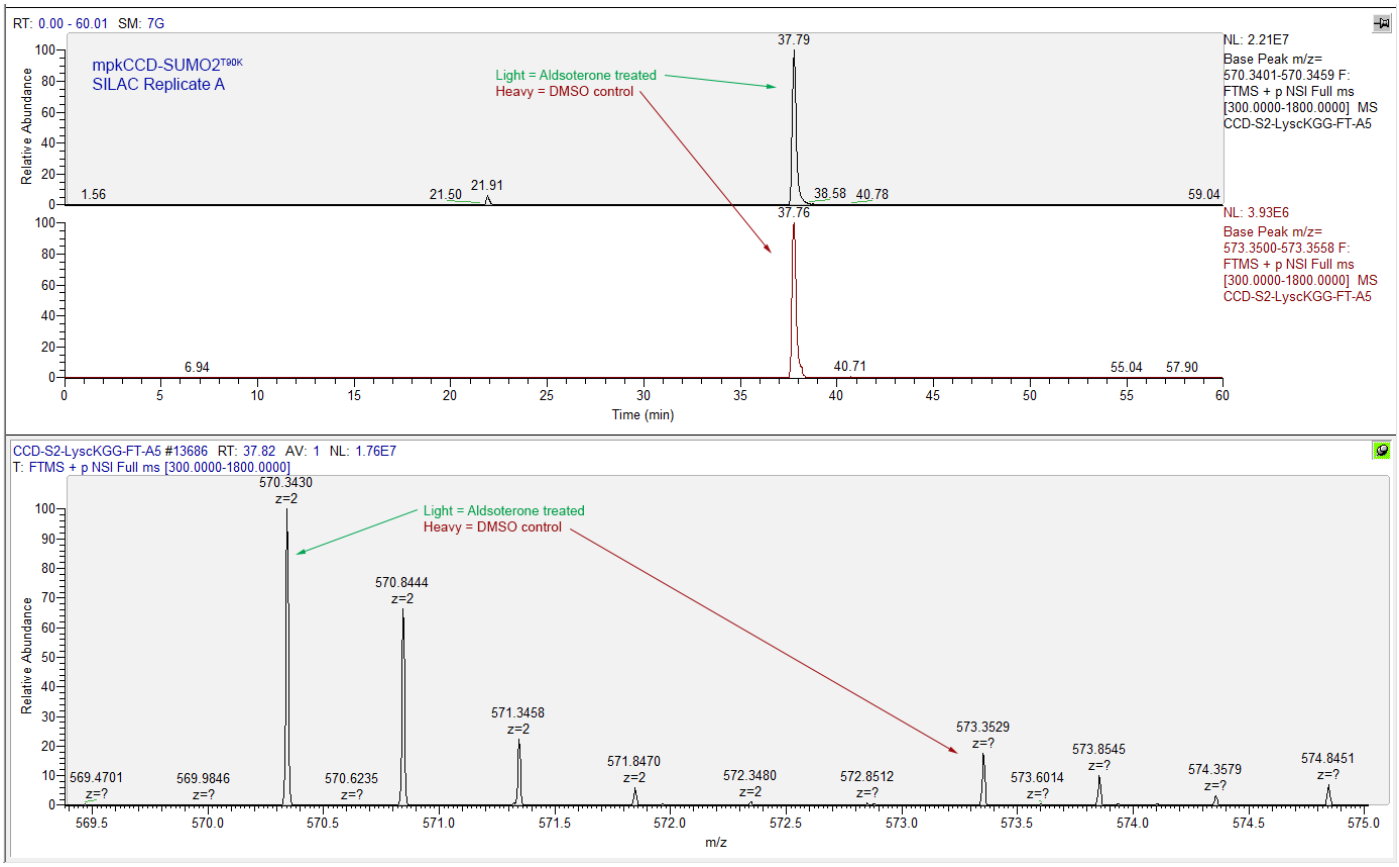
Relative Abundance
m/z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mpkCCD-SUMO2T90K
SILAC Replicate A
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Heavy = DMSO control
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Base Peak m/z= 570.3401-570.3459 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S2-LyscKGG-FT-A5
NL: 3.93E6
Base Peak m/z= 573.3500-573.3558 F: FTMS + p NSI Full ms [300.0000-1800.0000] MS CCD-S2-LyscKGG-FT-A5
Relative Abundance
Time (min)
CCD-S2-LyscKGG-FT-A5 #13686 RT: 37.82 AV: 1 NL: 1.76E7
T: FTMS + p NSI Full ms [300.0000-1800.0000]
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570.8444 z=2
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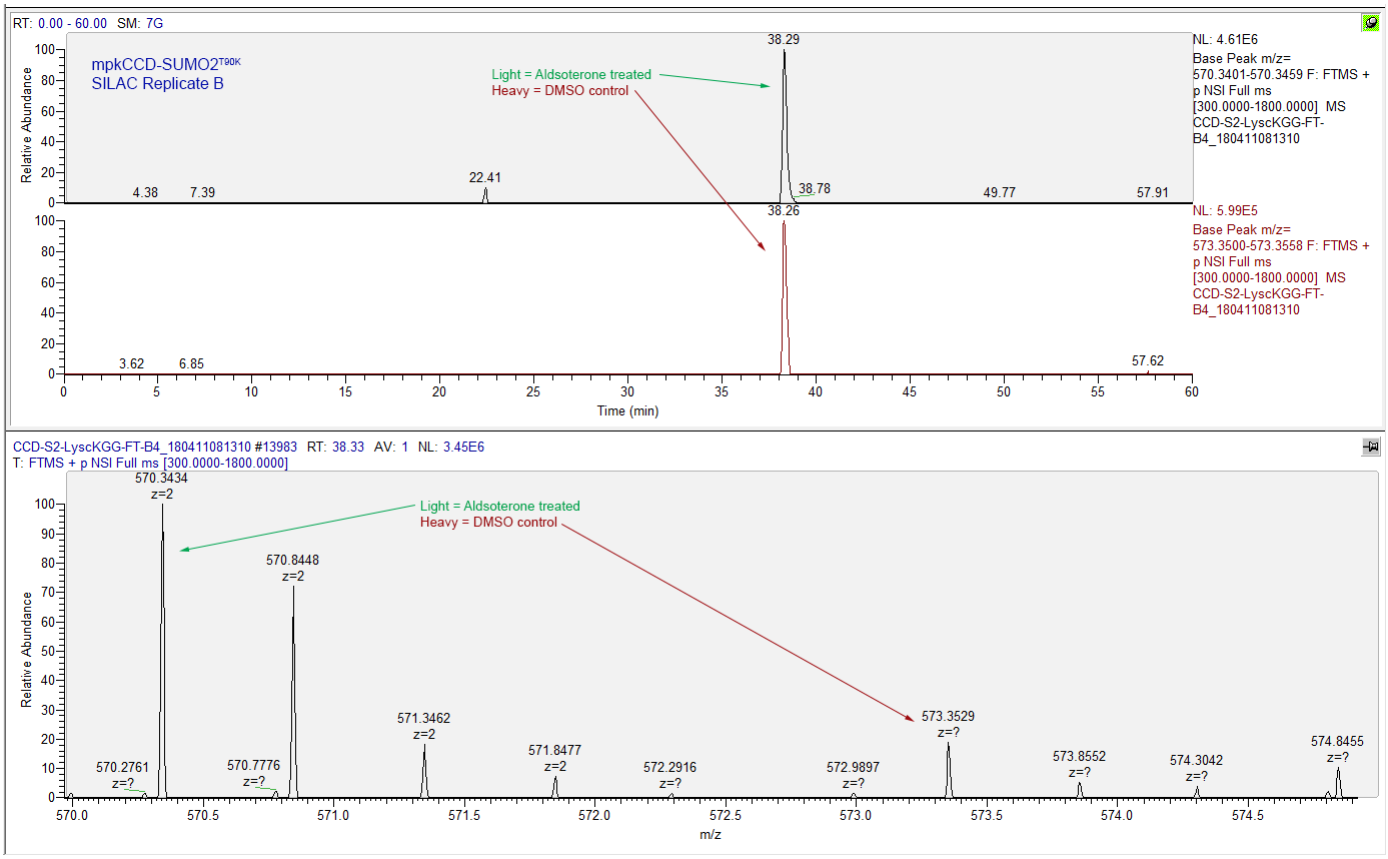
573.8545 z=?
574.8451 z=?
m/z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SILAC Replicate B
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Heavy = DMSO control
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NL: 5.99E5
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Relative Abundance
Time (min)
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m/z

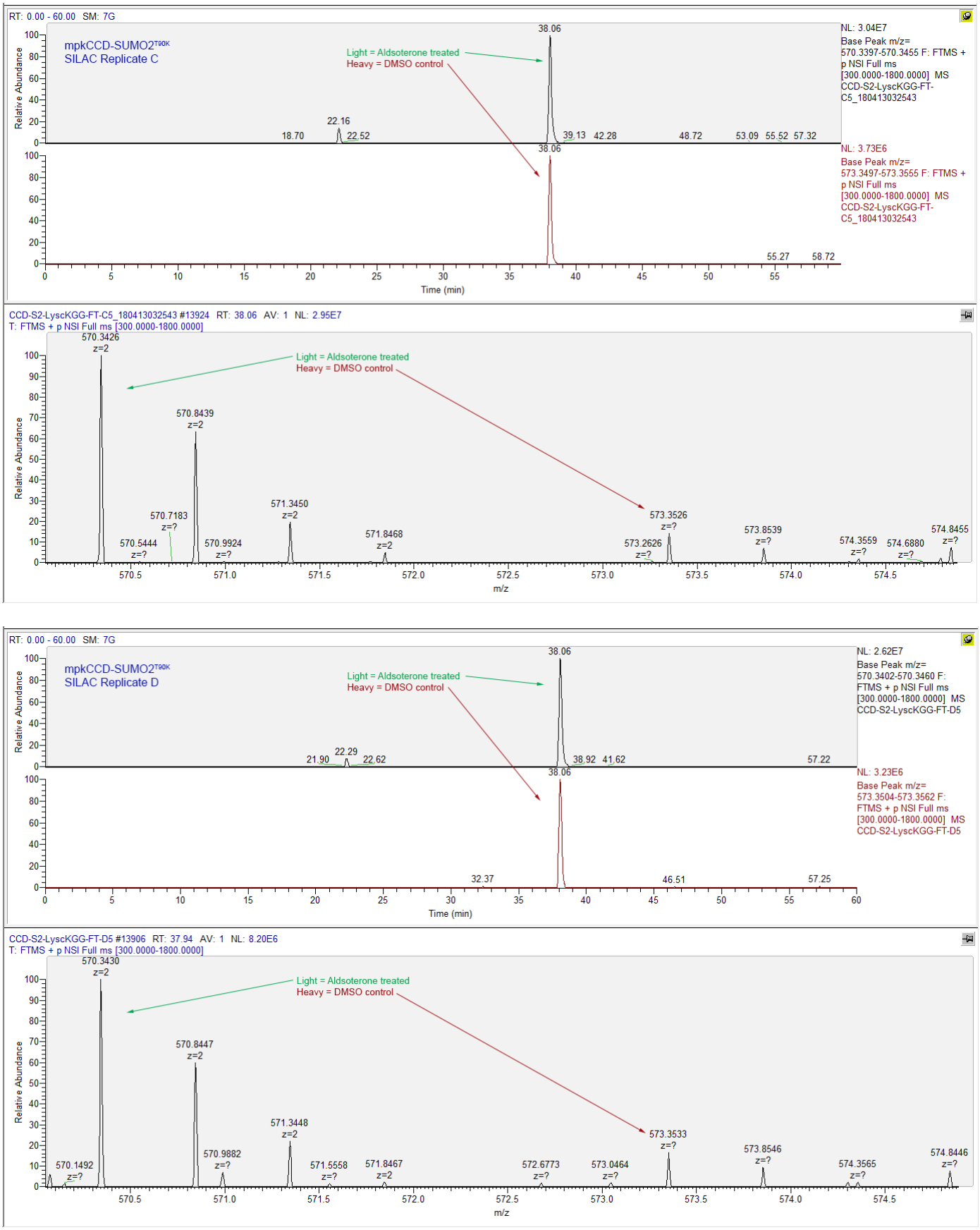
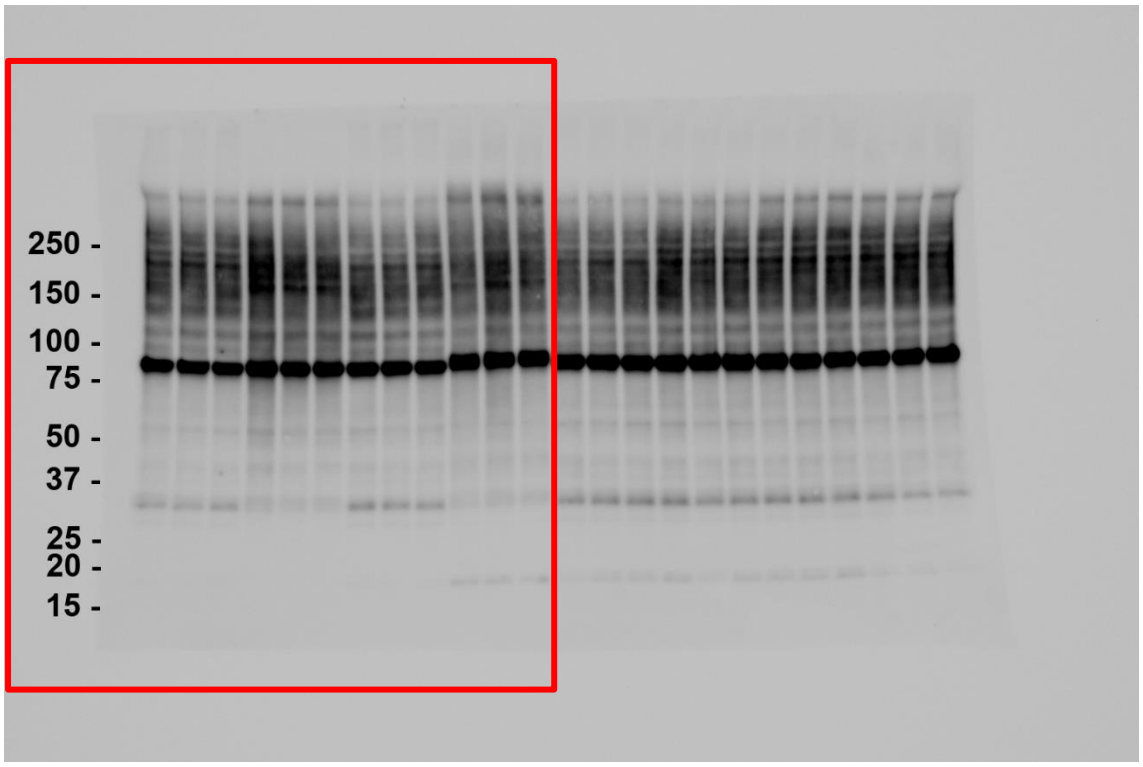


Figure S4. Example chromatogram of Pept2. Pept2 peptide chromatogram from each of the seven quantifiable SILAC experiments.

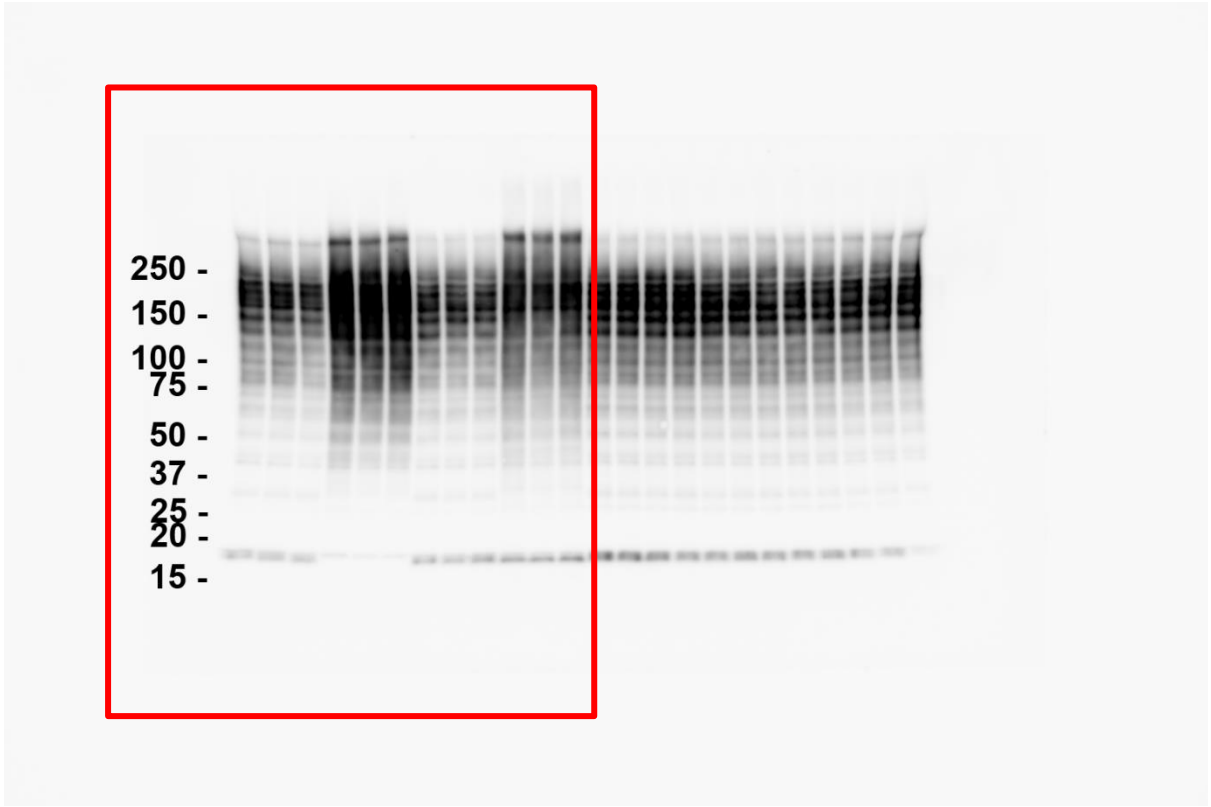
ANNEX: All included western blots in this file consist of the entire membrane that was incubated with the antibody/developed with ECL. Areas used in specific figures are highlighted.

Figure 1A – mpkCCD14-WT

SUMO1



SUMO2



Actin

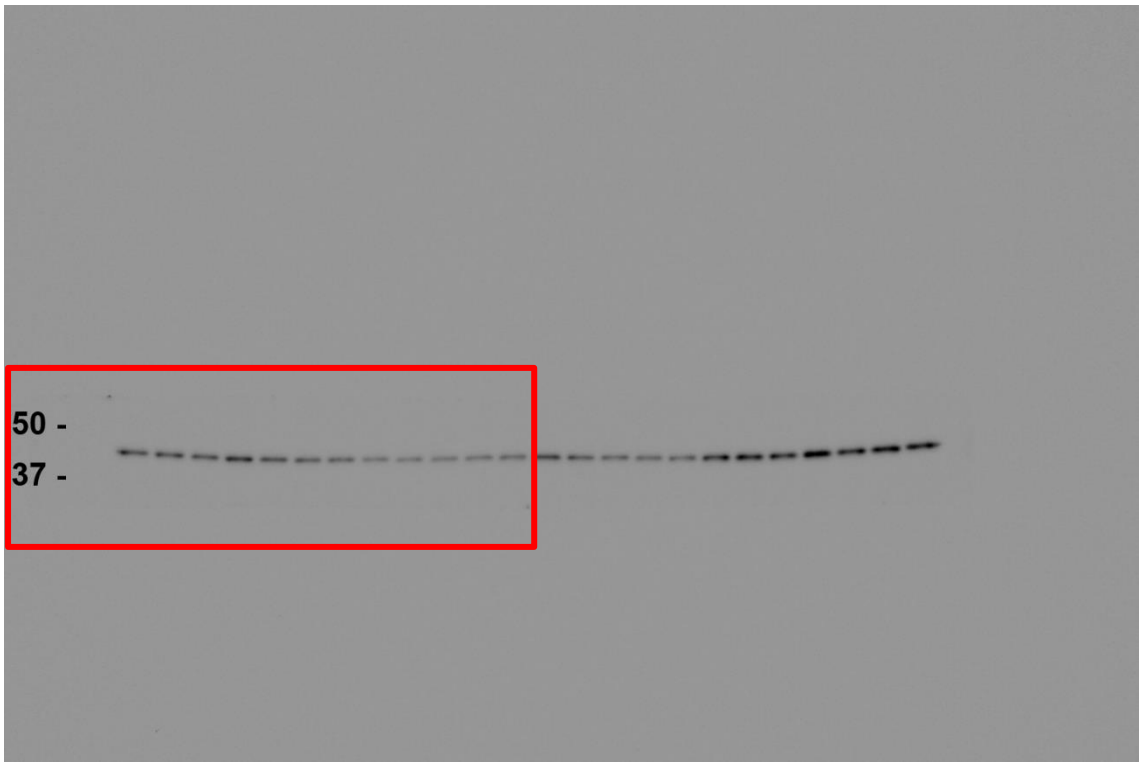
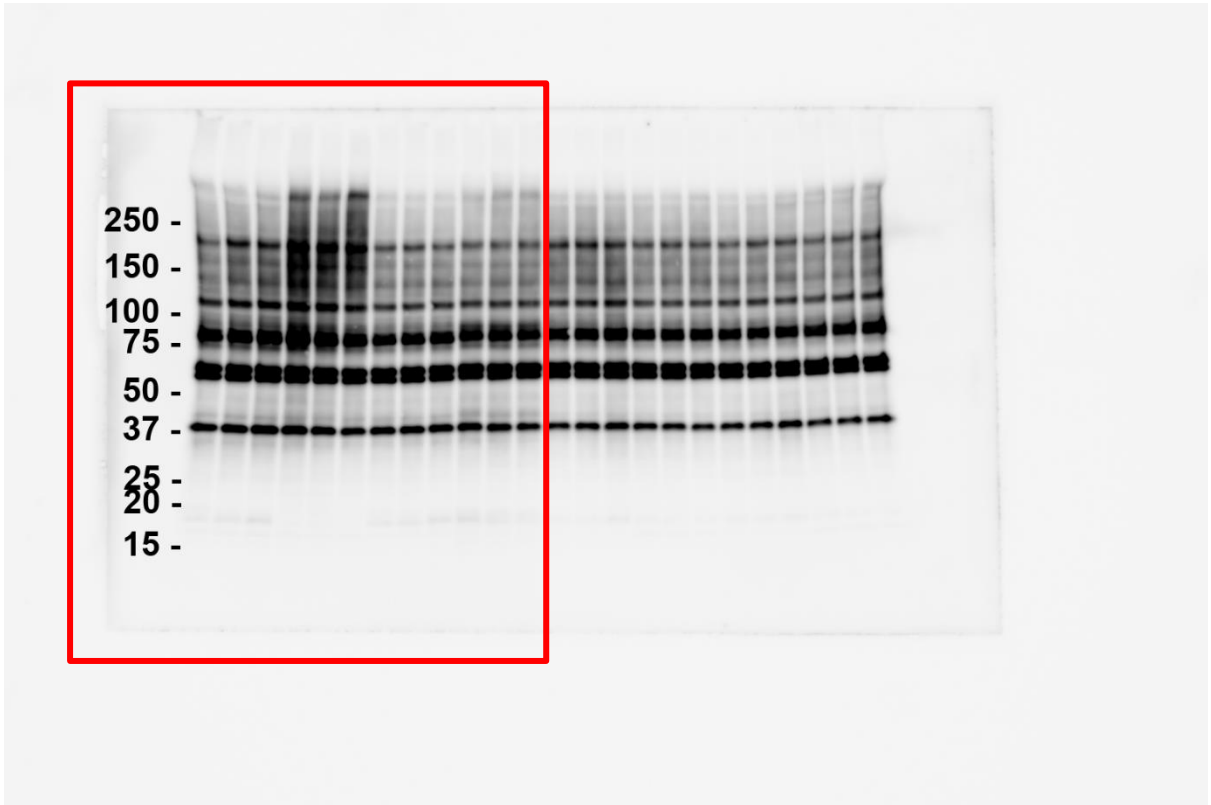
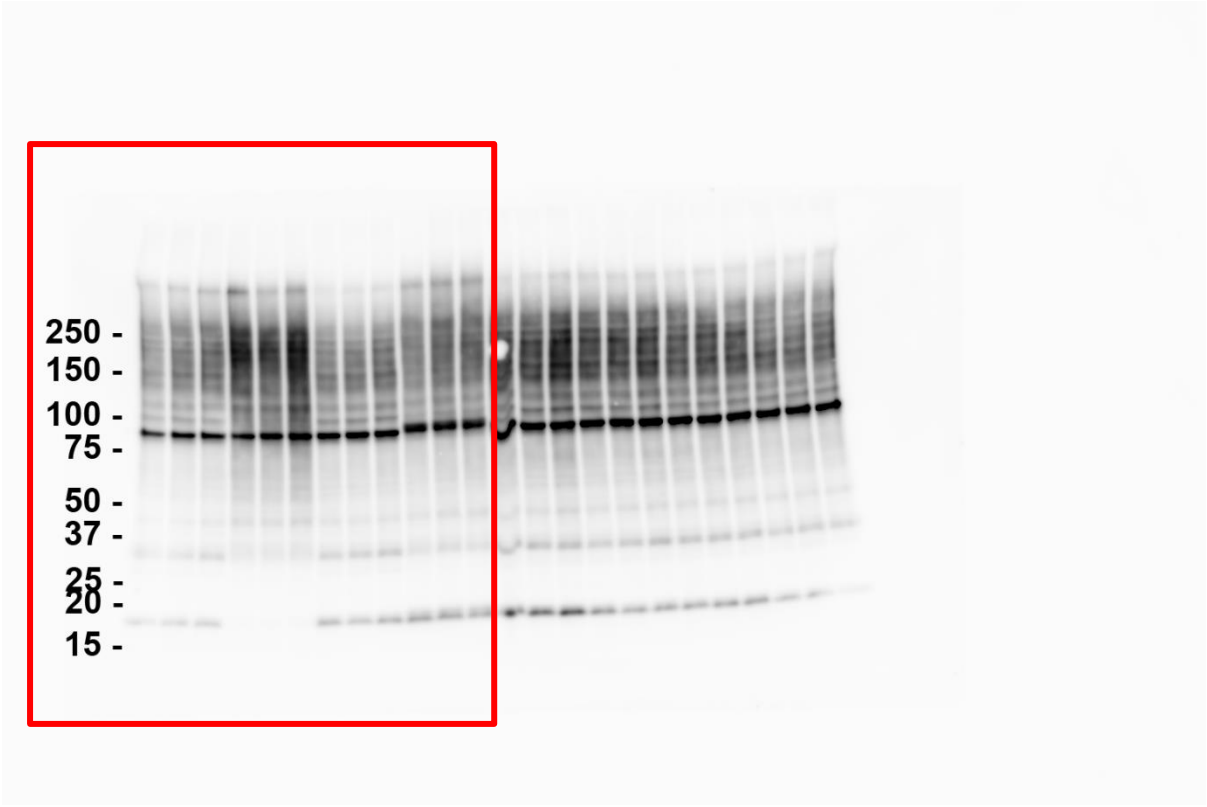


Figure 1B – mpkCCD SUMO1^{T95K}

6xHis



SUMO1



Proteasome 20s

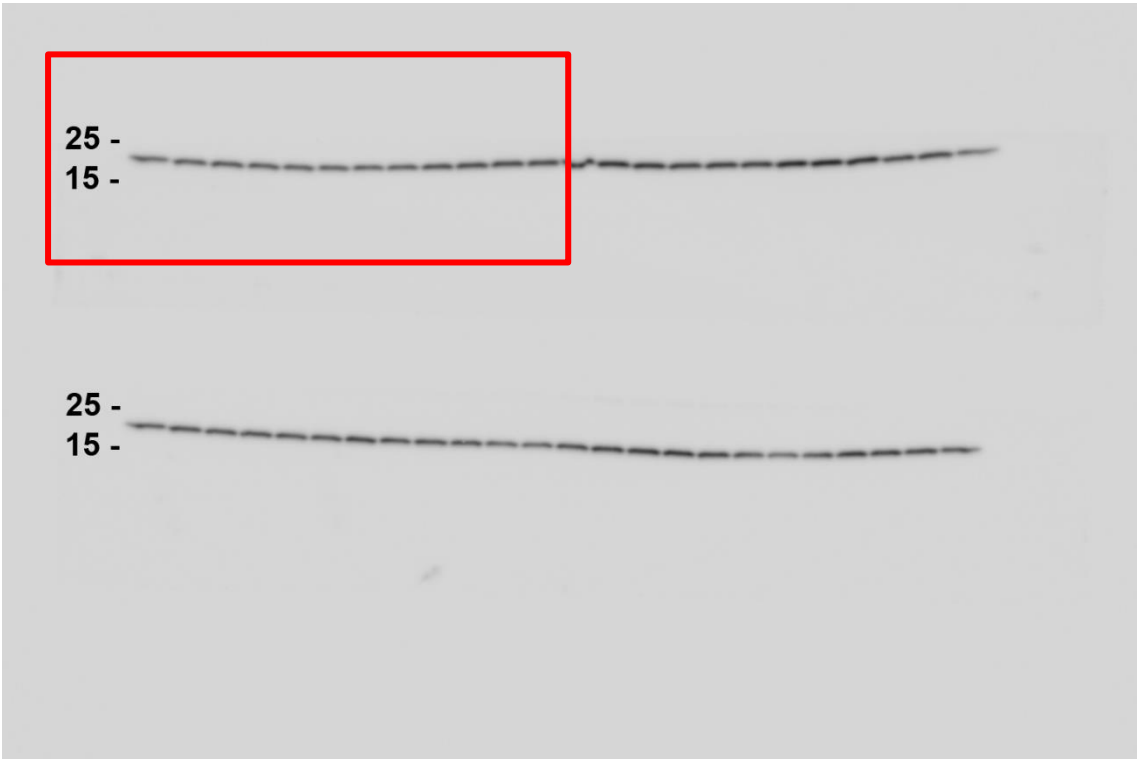
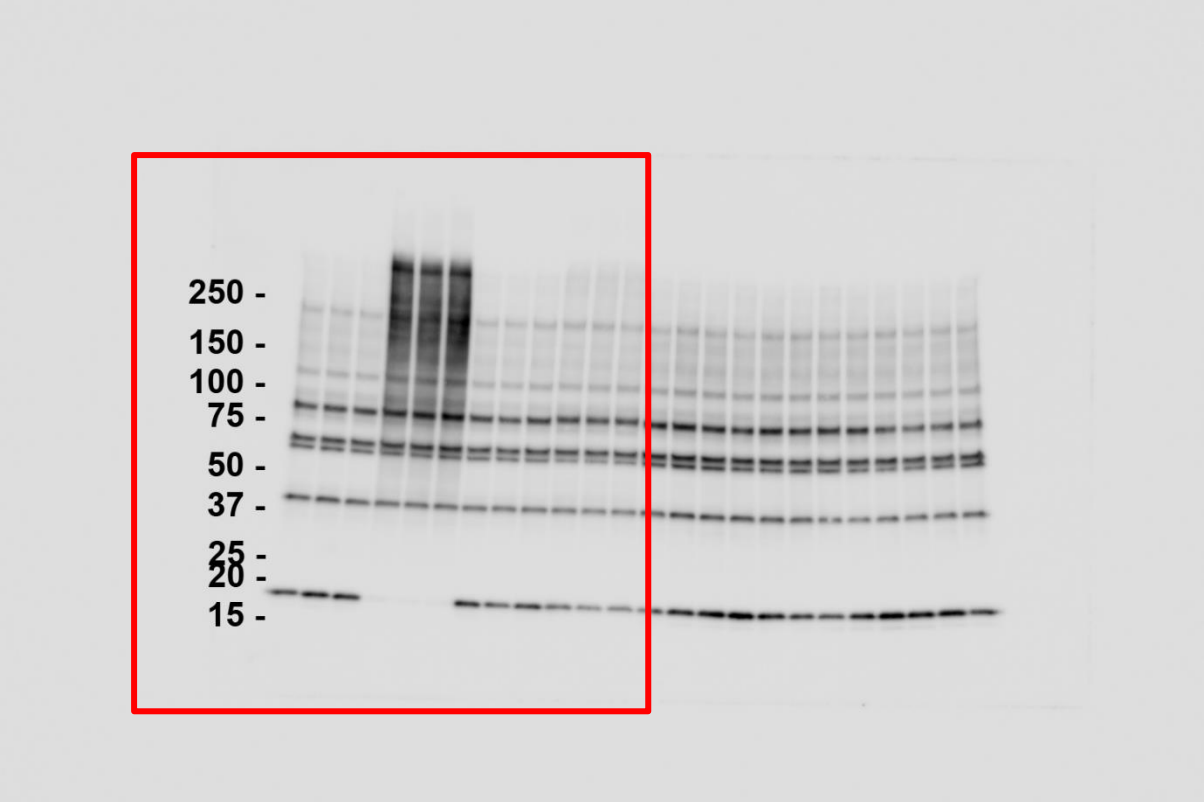
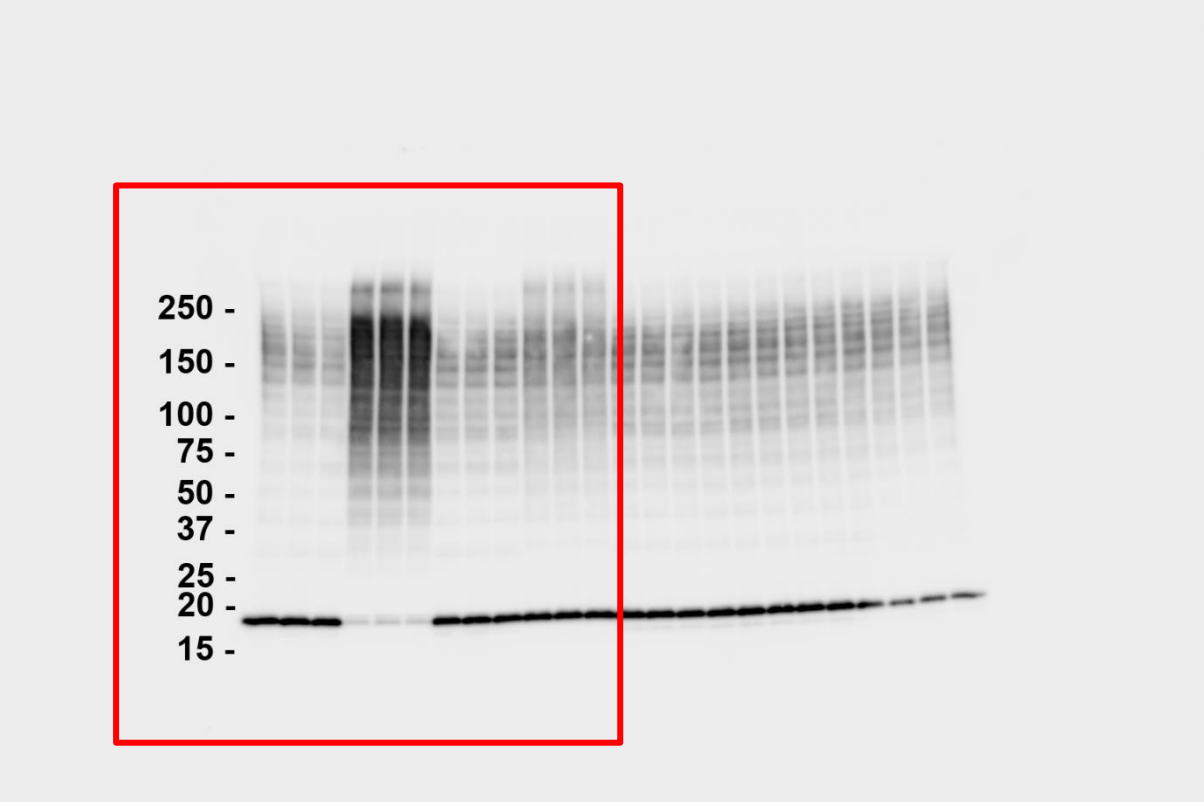


Figure 1C – mpkCCD-SUMO2^{T90K}

6xHis



SUMO2



Proteasome 20s

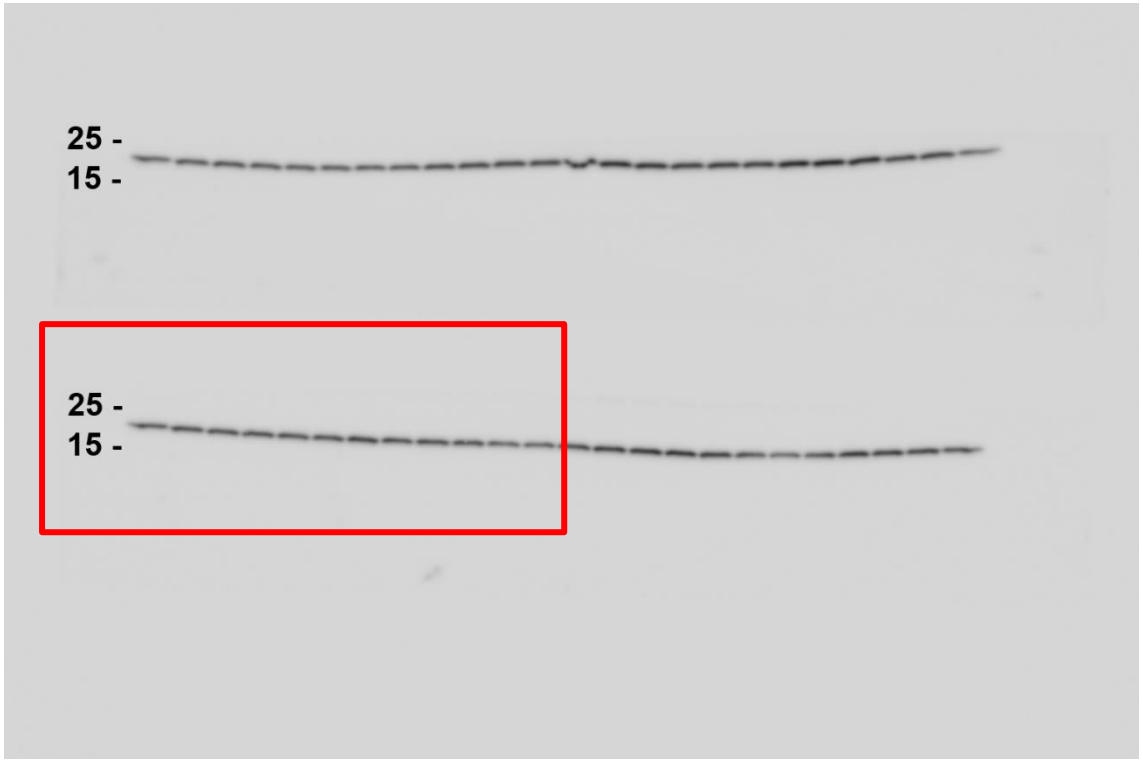


Figure 2 – panel A SUMO1

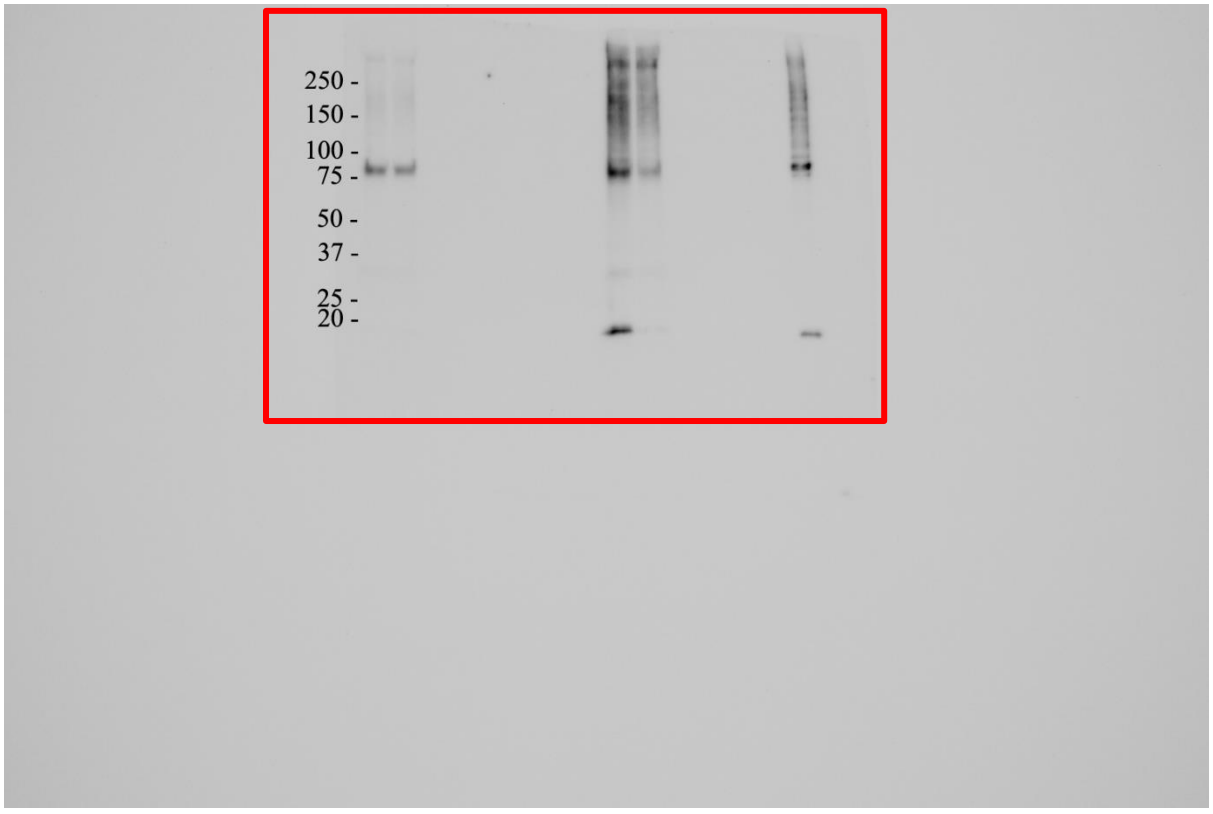


Figure 2 – panel A Coomassie

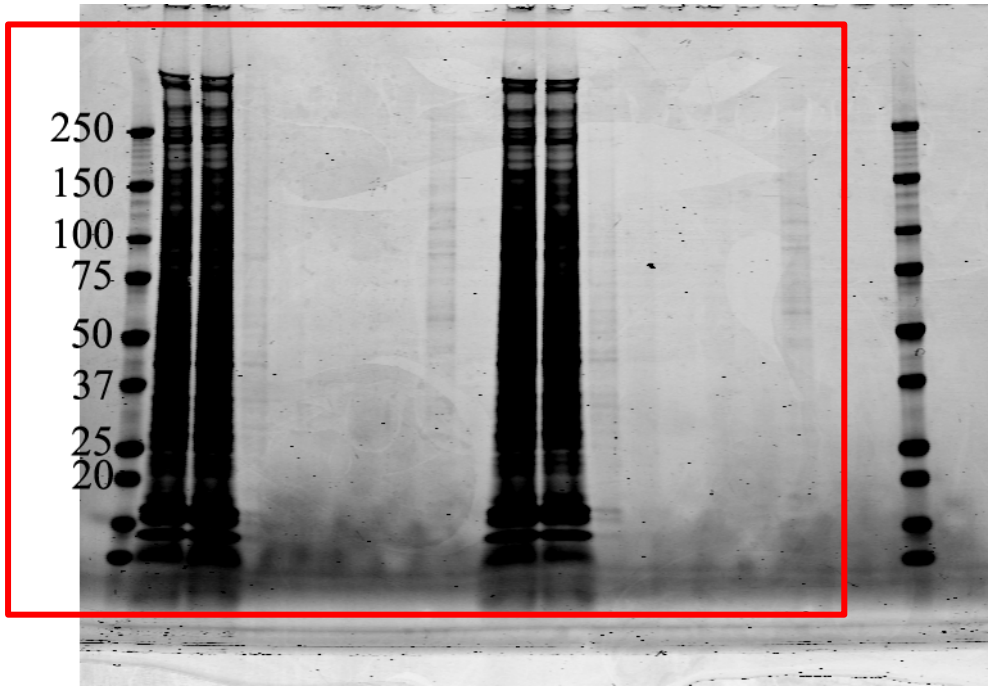
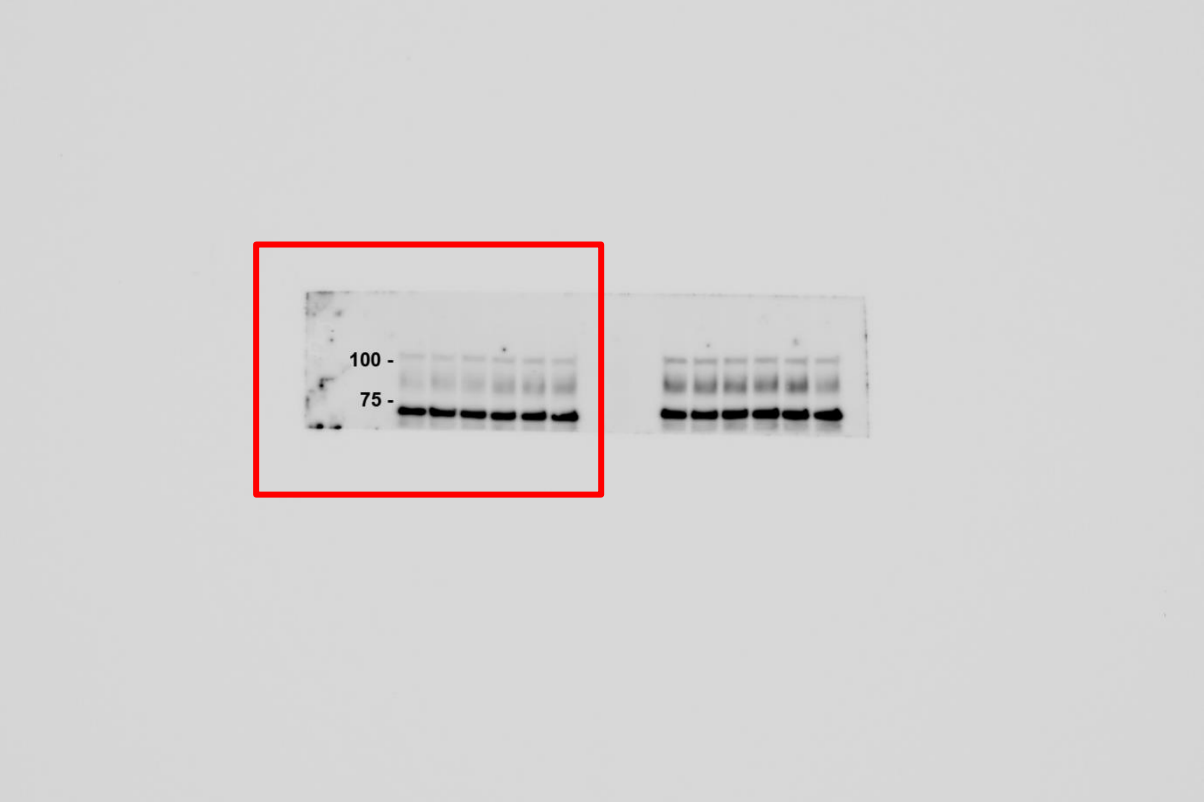


Figure 6 panel B

PEPT2



Actin

Figure S1 – panel A SUMO1 (Left heatshock and right M+P)

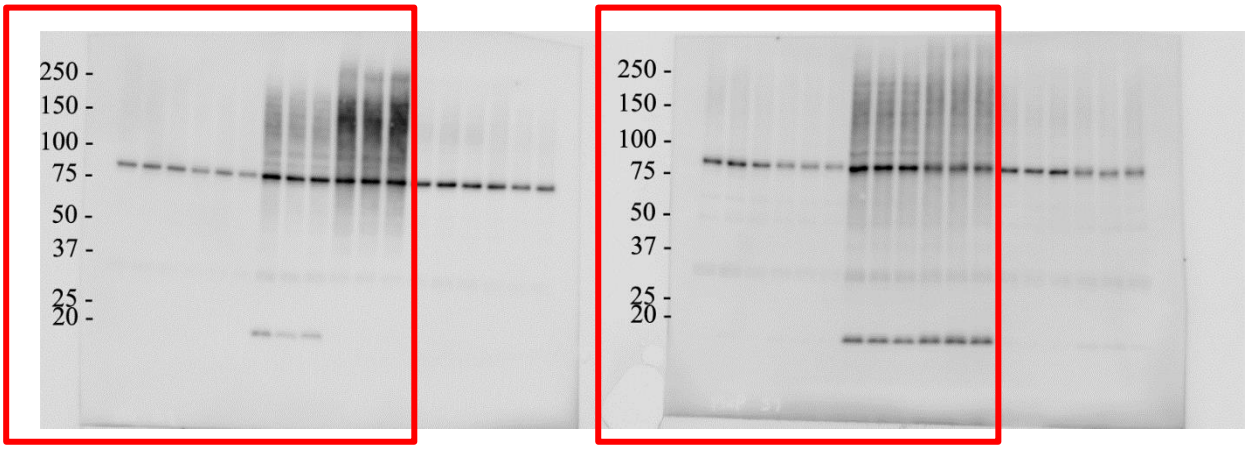
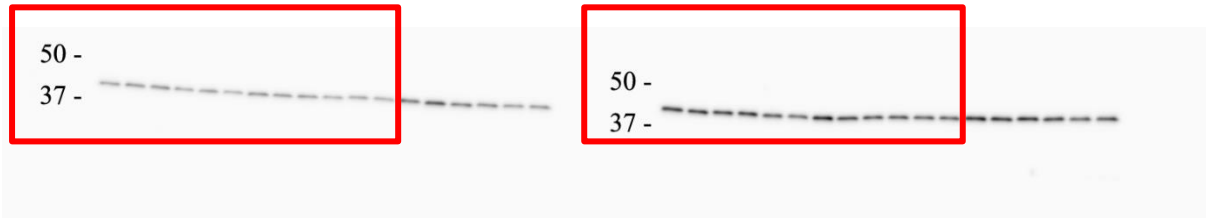


Figure S1 – panel A Actin (Left heatshock and right M+P)



Membrane has been cut before antibody incubation, so this is the entire membrane for Actin (Applies for all Actin or Proteasome 20s blots).

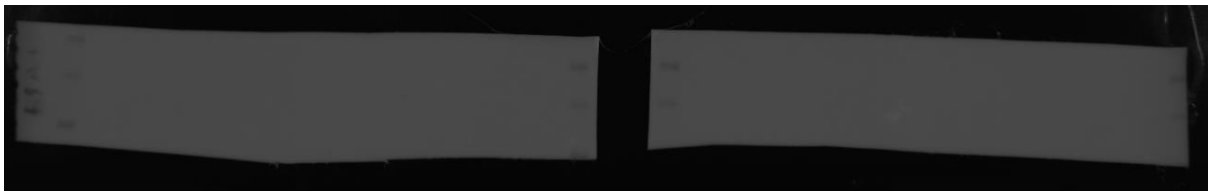


Figure S1 – panel B SUMO2 (M+P)

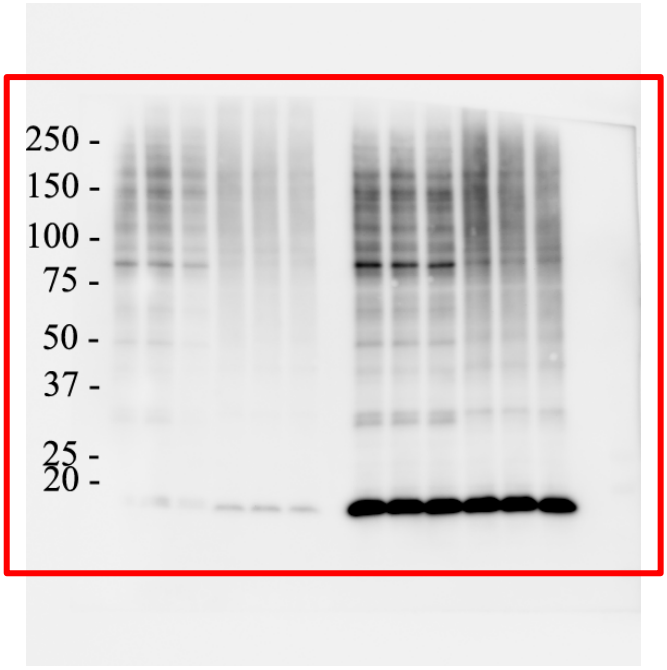


Figure S1 – panel B SUMO2 (Heatshock)

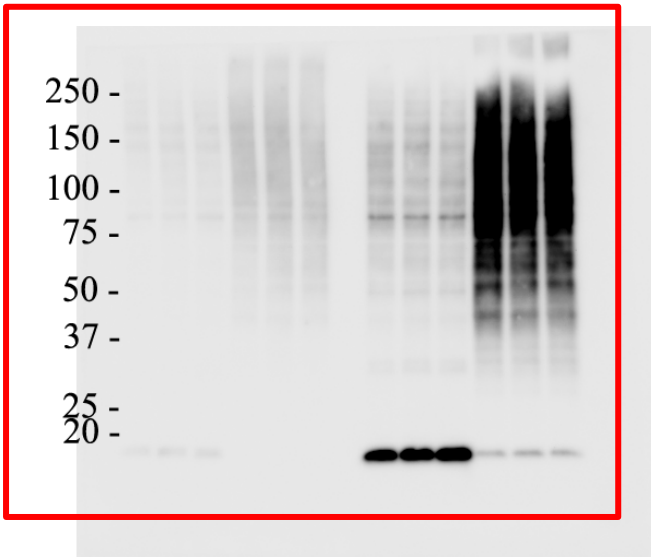


Figure S1 – panel B Actin (Left heatshock and right M+P)

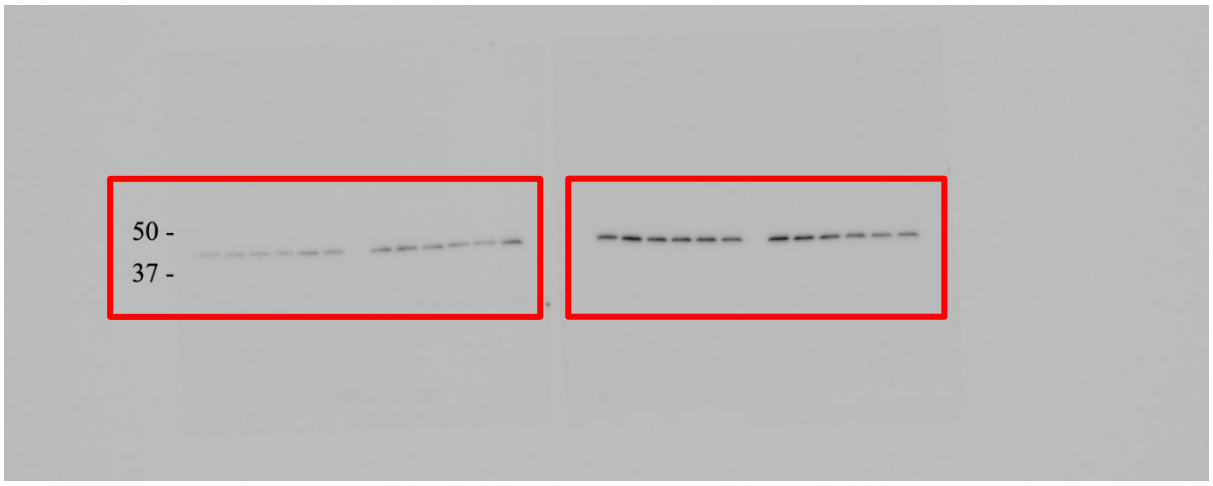
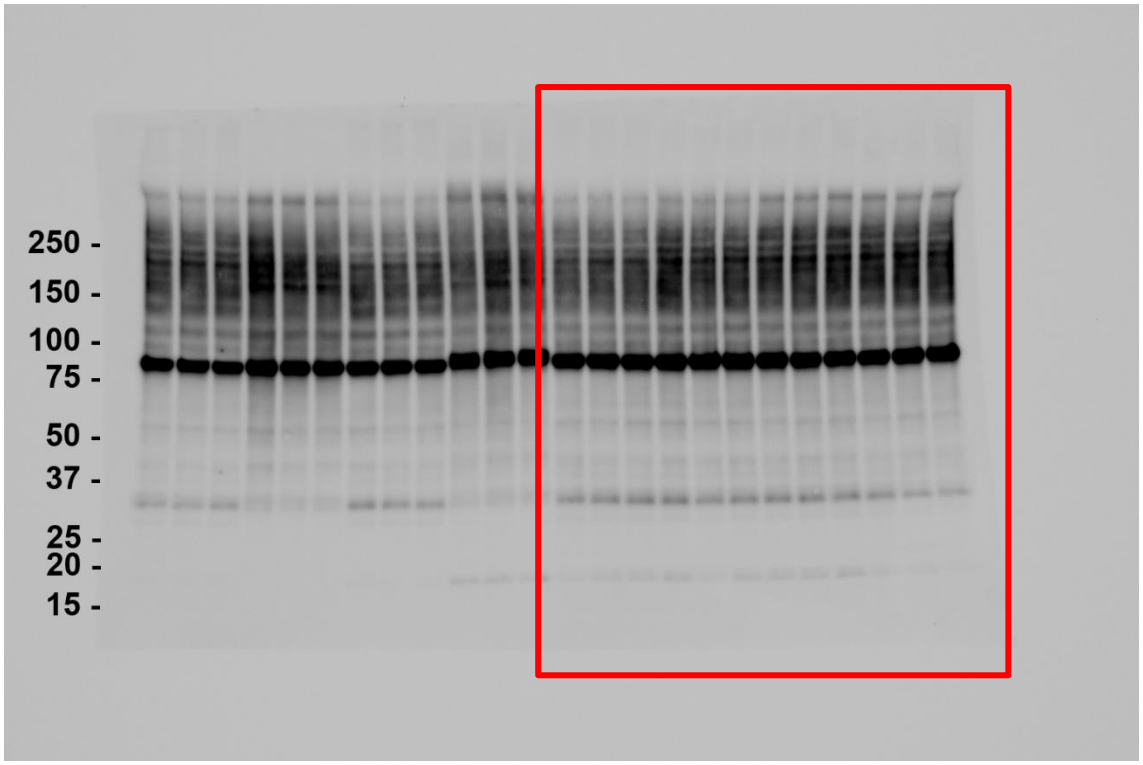
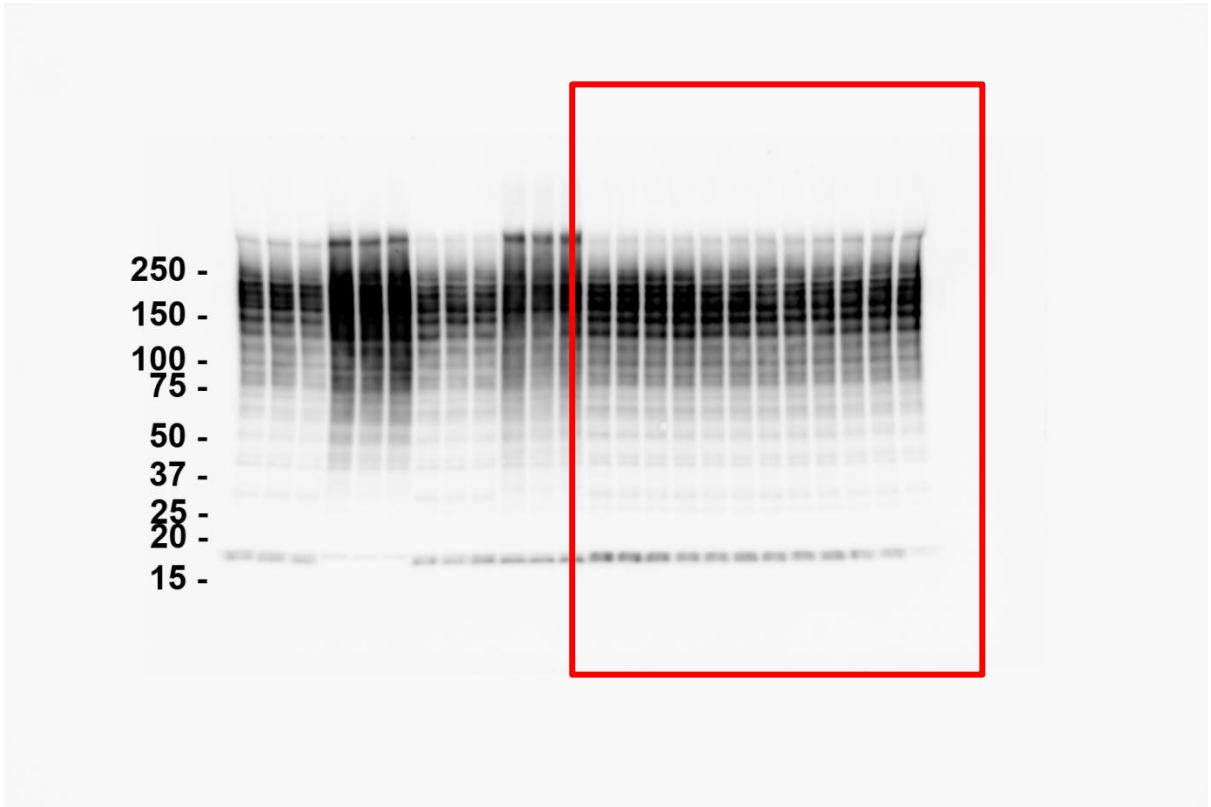


Figure S2 – mpkCCD14-WT

SUMO1



SUMO2



Actin

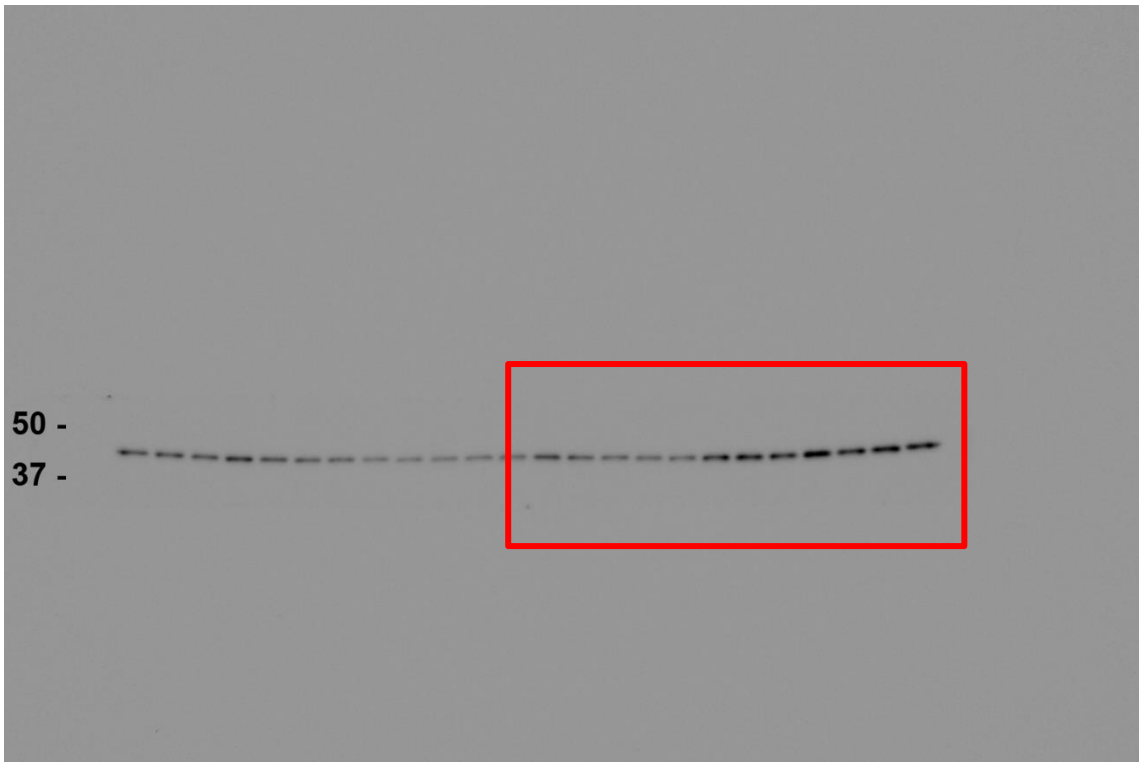
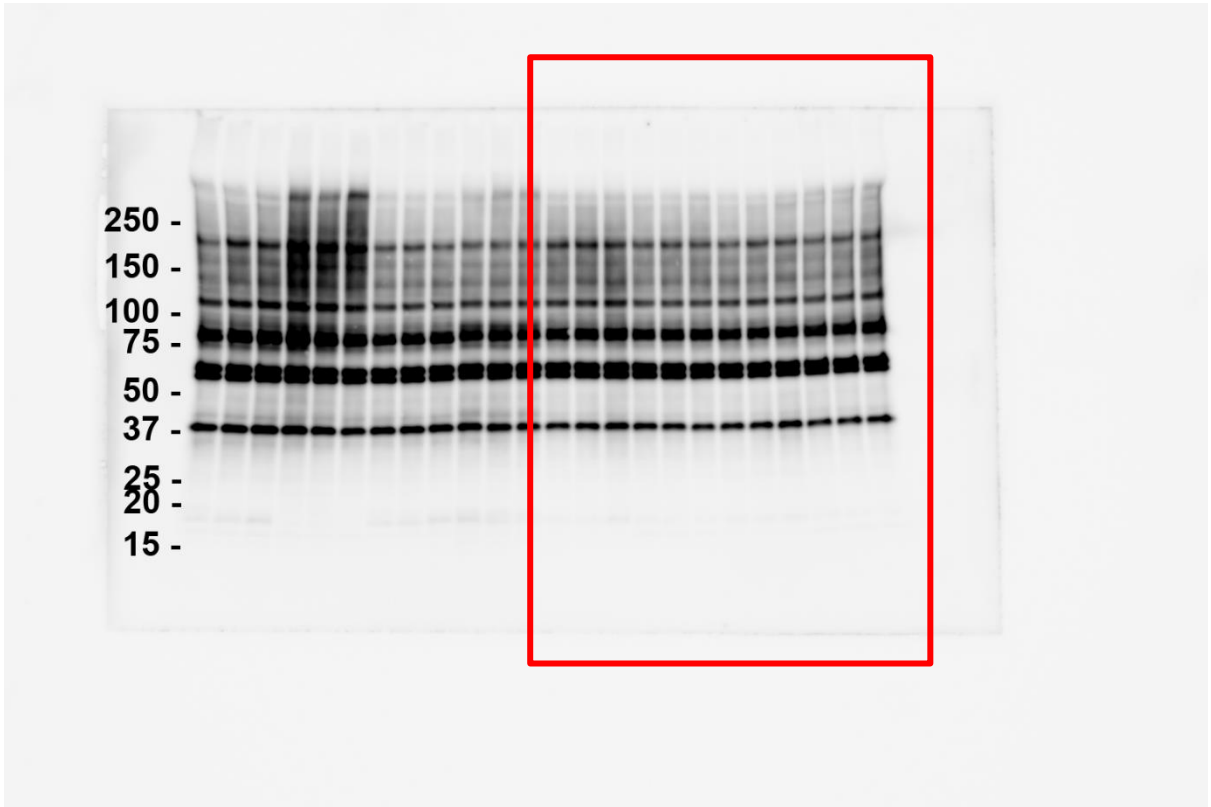
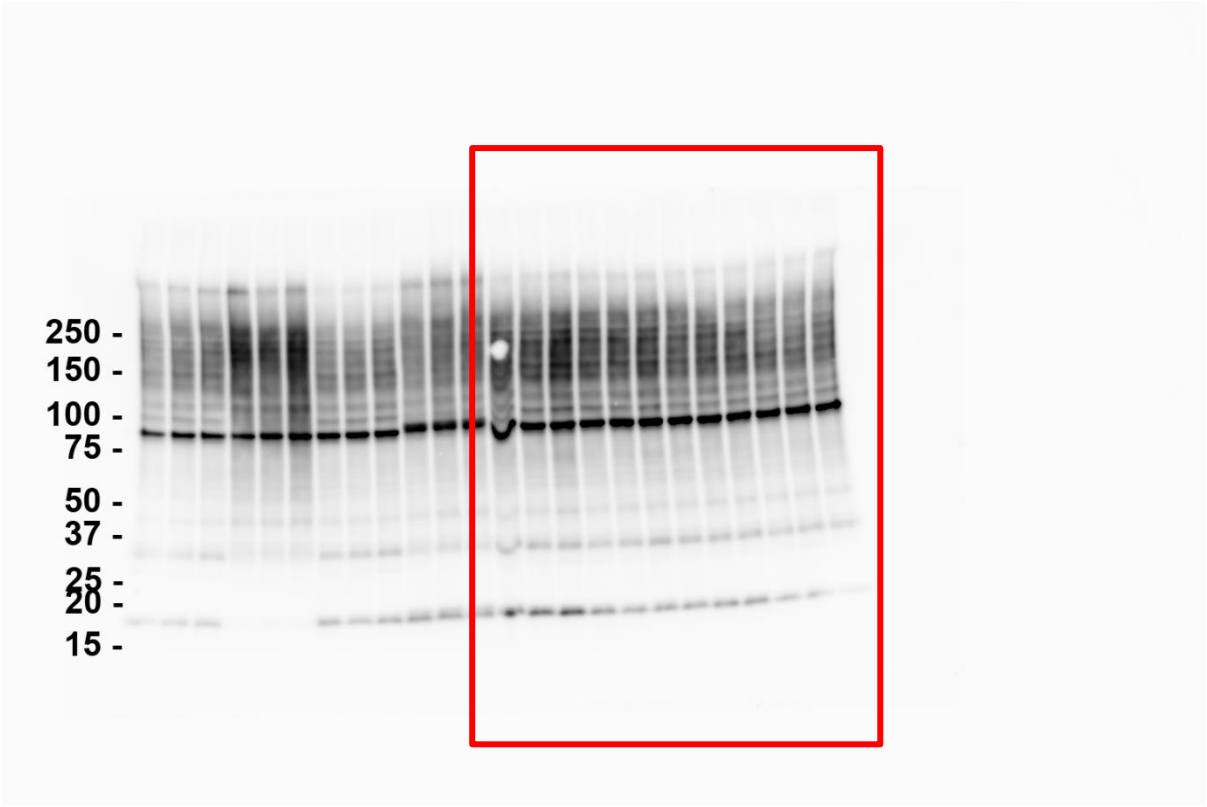


Figure S2 – mpkCCD SUMO1^{T95K}

6xHis



SUMO1



Proteasome 20s

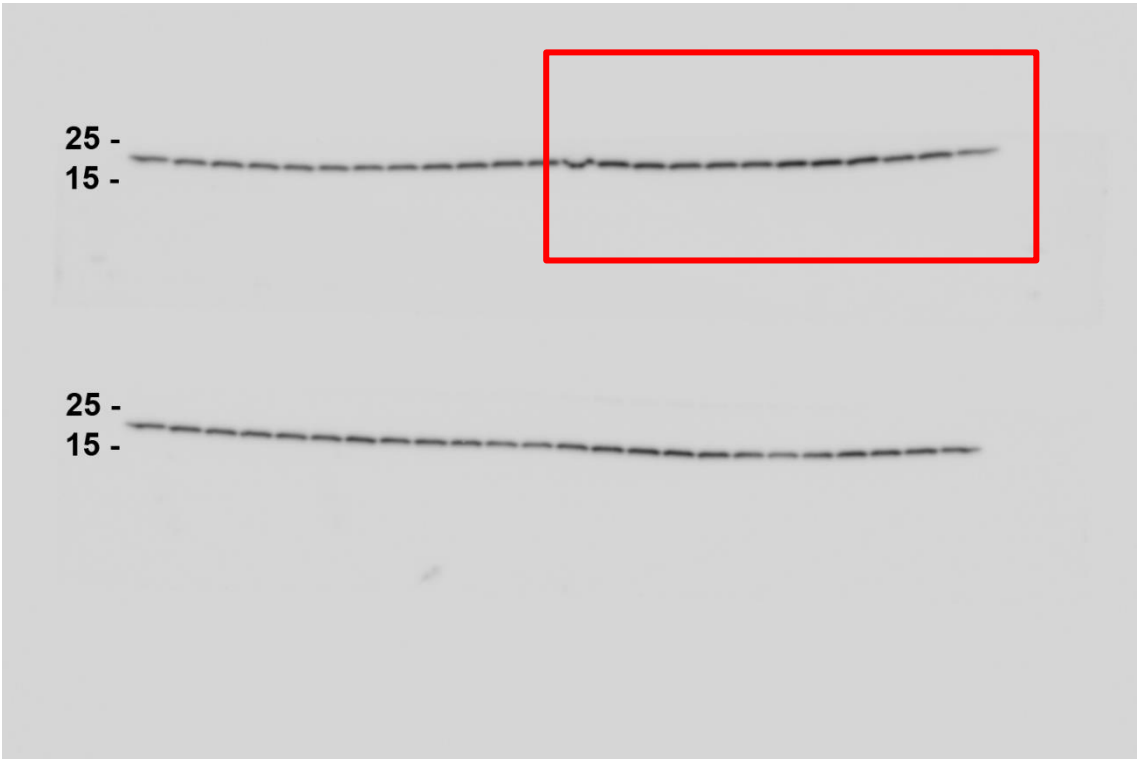
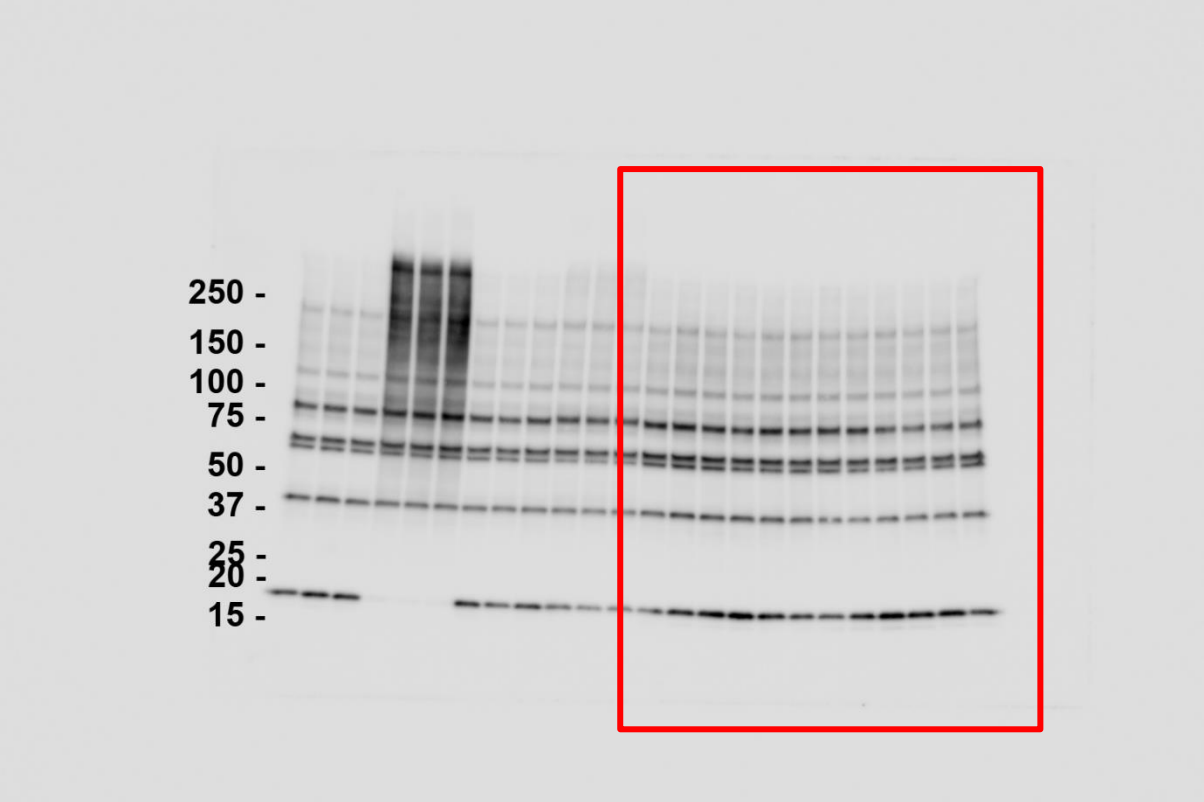
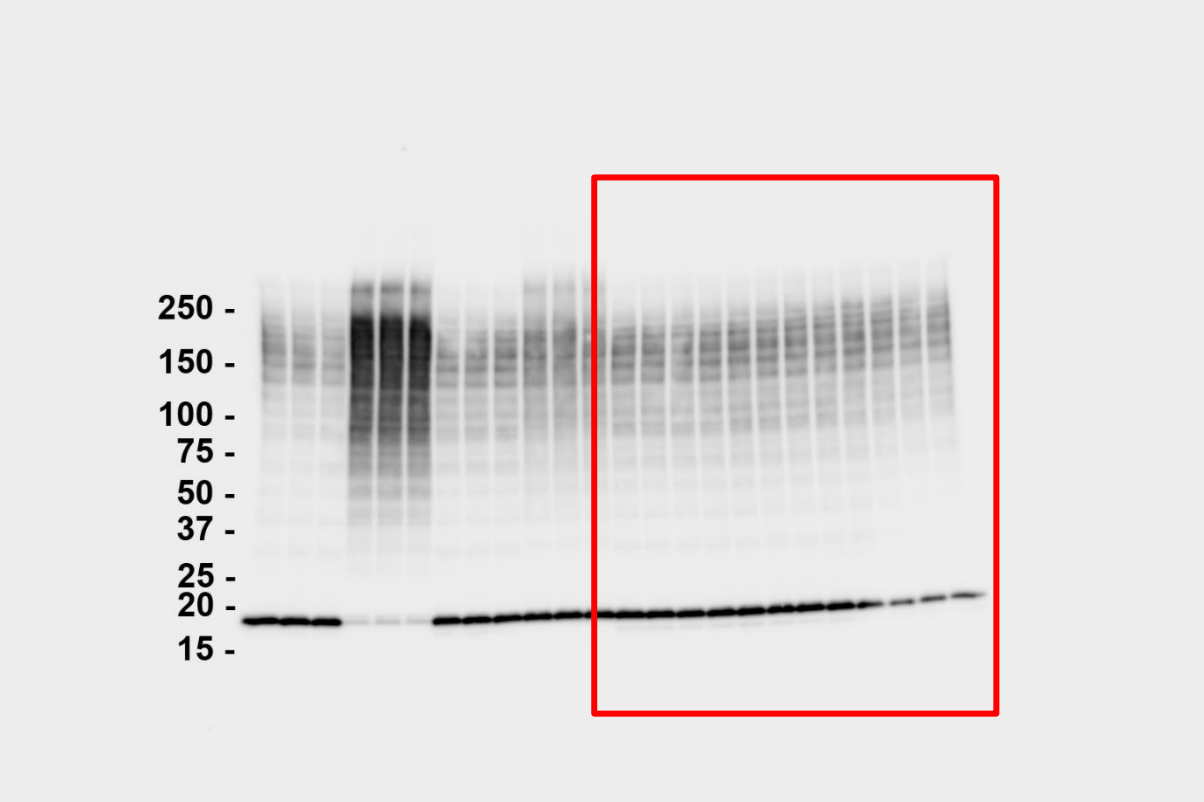


Figure S2 – mpkCCD-SUMO2^{T90K}

6xHis



SUMO2



Proteasome 20s