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SUPPLEMENTARY INFORMATION

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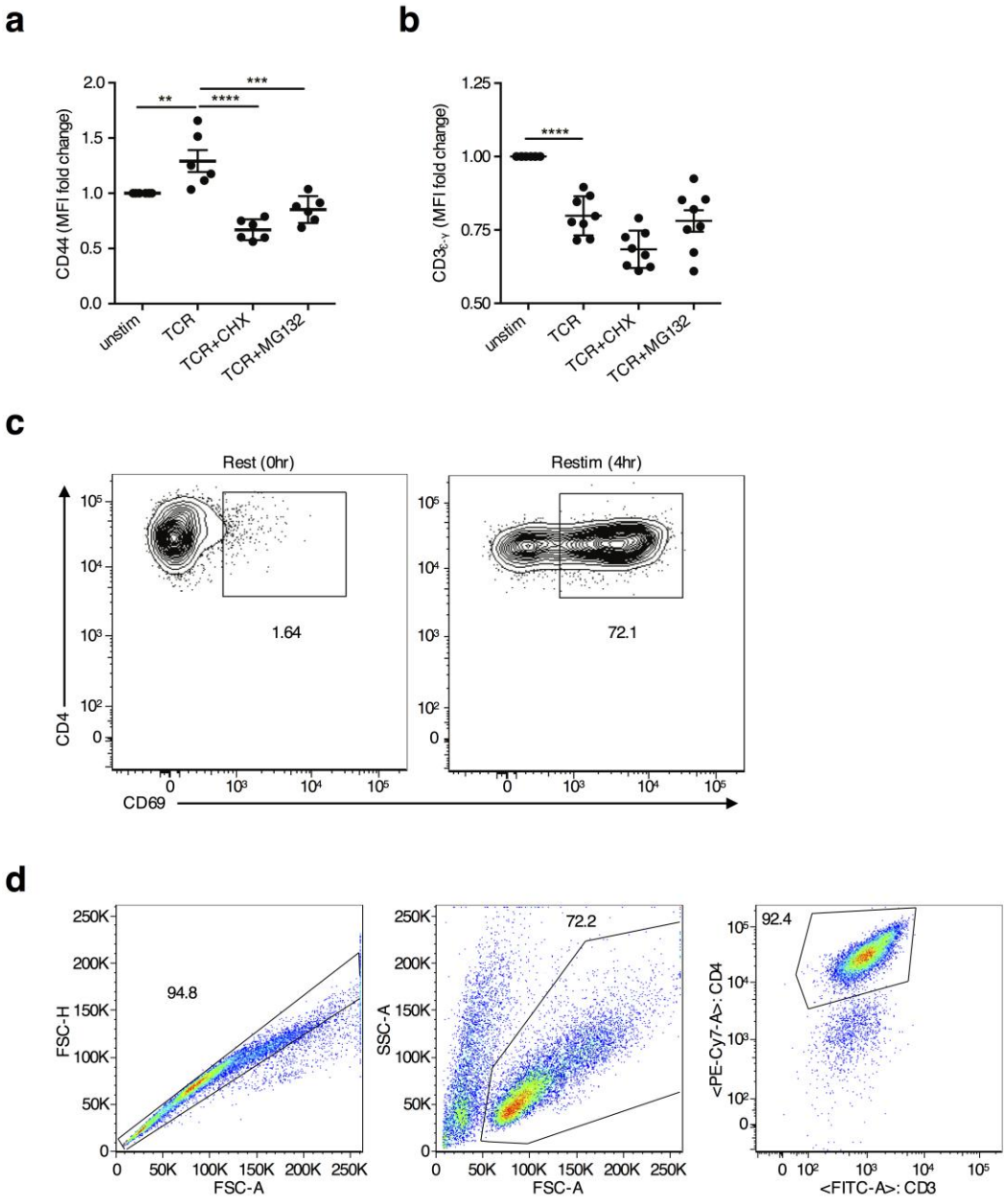
Integrative proteomics reveals an increase in non-degradative ubiquitylation in activated CD4$^+$ T cells

Joseph M. Dybas[1,2,3,5], Claire E. O'Leary[2,5], Hua Ding[2], Lynn A. Spruce[2], Steven H. Seeholzer[2] and Paula M. Oliver[1,2,4*]

[1]Division of Protective Immunity, Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA, USA. [2]Cell Pathology Division, Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA, USA. [3]Department of Biomedical and Health Informatics, The Children's Hospital of Philadelphia, Philadelphia, PA, USA. [4]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA. [5]These authors contributed equally: J. M. Dybas, C. E. O'Leary. *e-mail: paulao@pennmedicine.upenn.edu

Supplementary Figure 1



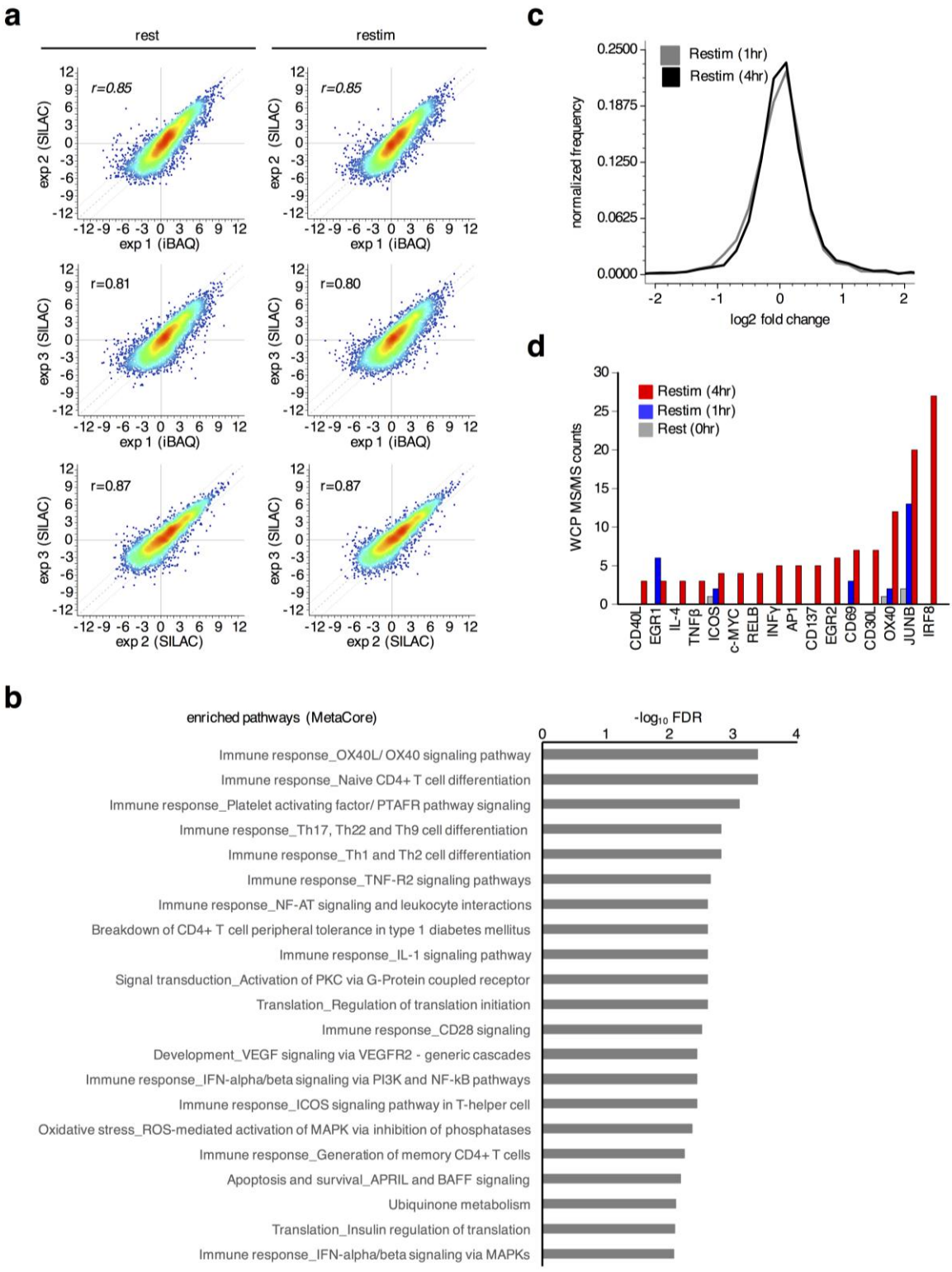
Supplementary Figure 1

Proteasome inhibitor MG132 prevents appropriate T cell activation.

a, Flow cytometry measurement of surface CD44 mean fluorescence intensity (MFI) fold change during TCR stimulation in $CD4^+$ T cells rested or restimulated for 4 hours with CD3+CD28 antibody coated beads (1:1 cell:bead ratio). Restimulated cells were untreated or treated with MG132 or cycloheximide (CHX) for the entire 4 hour stimulation. **b**, Flow cytometry measurement of surface CD3ε-γ MFI fold change during TCR stimulation in CD4+ T cells, as described in (**a**). a-b MFI fold changes are normalized to intensity in unstimulated T cells within the same experiment. Data are compiled from 2 independent experiments comprising 8 mice, mean shown

+/- SEM. P values calculated by one-way ANOVA with Holm–Sidak test for multiple comparisons, **P<0.01***P<0.001, ****P<0.0001. **c**, Flow diagram showing expression of surface CD69 in $CD4^+$ T cells rested or restimulated for 4 hours using CD3+CD28 antibody coated beads (3:1 cell:bead ratio). A representative plot of activation achieved in three WCP experiments is shown (minimum 65% activation across three experiments). Previously gated on live singlets, CD3+CD4+. **d**, Gating strategy for flow cytometry analysis.

Supplementary Figure 2

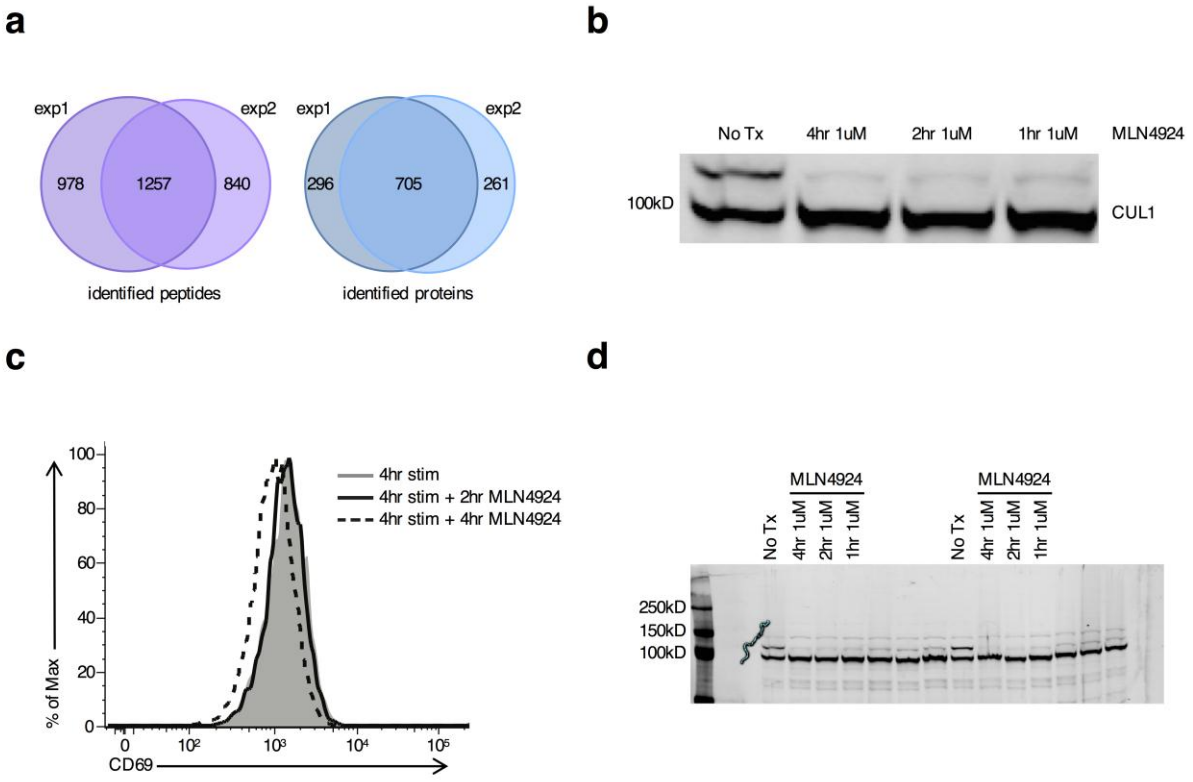


Supplementary Figure 2

Whole cell proteomics of CD4+ T cell during TCR activation identifies upregulation of TCR associated proteins and pathways.

a, Pairwise comparisons of protein abundance measured from WCP mass spectrometry experiments in $CD4^+$ T cells rested or restimulated for 4 hours with CD3+CD28 antibody coated beads (3:1 cell:bead ratio).. For label free quantification (exp 1), log_2 normalized iBAQ values were used to represent protein abundance at rest or restimulation. For SILAC quantification (exp 2 and 3), log_2 normalized "heavy" intensity was used for the resting cell protein abundance and "light" intensity was used for restimulated cell protein abundance. Correlation coefficients were calculated for all-by-all pairwise comparisons of three experiments, using the Pearson's method. **b**, MetaCore (portal.genego.com) pathway enrichment within the significantly upregulated proteins identified in the WCP mass spectrometry experiments in $CD4^+$ T cells unstimulated or stimulated, as described in (**a**). Analysis was performed on proteins exhibiting log_2 fold change > 0 and p-value < 0.05, based on a two-tailed Students-t test. Enriched pathways were identified by FDR, based on a q-value calculation, performed by the MetaCore program. **c**, Histograms of log_2 fold changes in protein abundance from WCP mass spectrometry experiments in CD4+ T cells unstimulated or stimulated, as described in (**a**). Log2 fold changes are compared for 1 hour stimulation (grey) and 4 hour stimulation (black). d, MS/MS counts from the label-free quantified WCP mass spectrometry experiments in CD4+ T cells rested or restimulated, as described in (**a**), for 1 or 4 hours. MS/MS counts are used as a measure of protein abundance for proteins known to be induced upon CD4+ T cell activation. Rest (0hr) and 4 hour data are reproduced from main Figure 2d to provide for comparison with 1 hour data.

Supplementary Figure 3

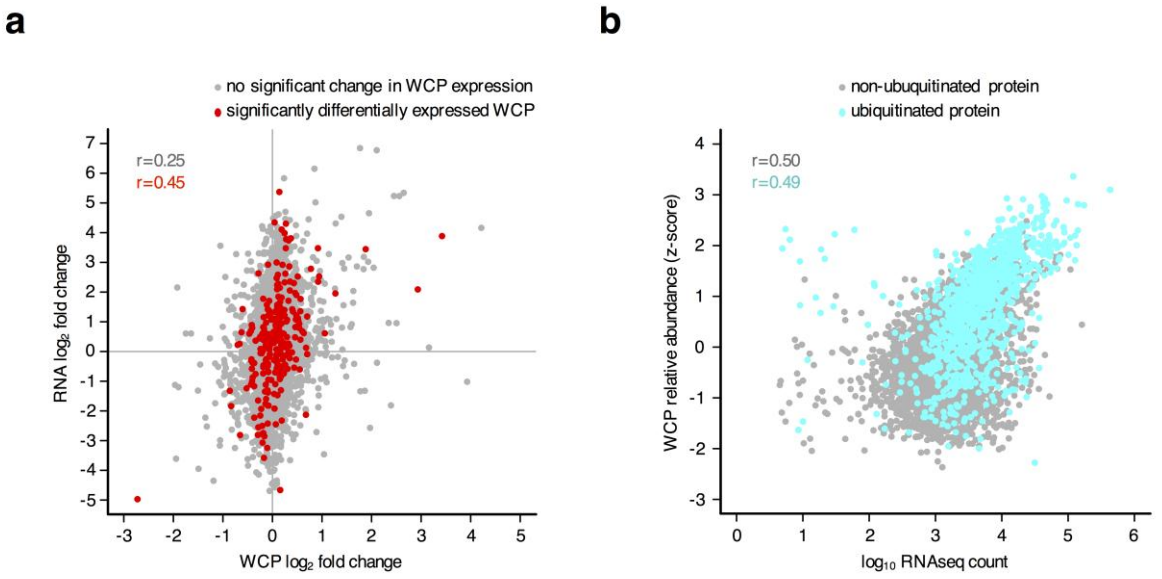


Supplementary Figure 3

Di-glycine remnant proteomics during TCR stimulation is negligibly impacted by neddylation inhibition.

a, Intersection of identified di-glycine remnant peptides (left) and associated proteins (right) in two independent di-glycine remnant mass spectrometry experiments in $CD4^+$ T cells unstimulated or stimulated for 4 hours with CD3+CD28 antibody coated beads (3:1 cell:bead ratio). **b**, Western blot showing cullin 1 protein abundance in CD4+ T cells unstimulated or stimulated, as described in (a), with 1 hour, 2 hour or 4 hour treatment with 1uM concentration of neddylation inhibitor MLN4924. With no drug, a prominent neddylation band is seen at higher molecular weight, along with the native Cul1 band. The neddylation band is reduced with addition of MLN4924 neddylation inhibitor, at dose and times indicated. A representative blot of cullin abundance observed in three indep endent experiments is shown (n=3 for addition of MLN4924 at 2 hours of 4 hour stimulation; for other time points n=2). **c**, Flow cytometry measurement of CD69 upregulation on CD4+ T cells unstimulated or stimulated for 4 hours with CD3+CD28 antibody coated beads (1:1 cell:bead ratio) and untreated (grey) or treated with addition of MLN4924 (1uM) during the final 2 hours (solid black line) or entire 4 hours (dashed black line) of the 4-hour stimulation. A representative plot of distributions observed in two (2 hour and 4 hour treatment) or three (4 hour treatment) independent experiments is shown. **d**, Image of the uncropped and unaltered blot obtained from the cullin 1 protein abundance experiment described in (**b**). The blot shows the lysates from two independent experiments run on a single gel (lanes 1-4 and 8-11).

Supplementary Figure 4

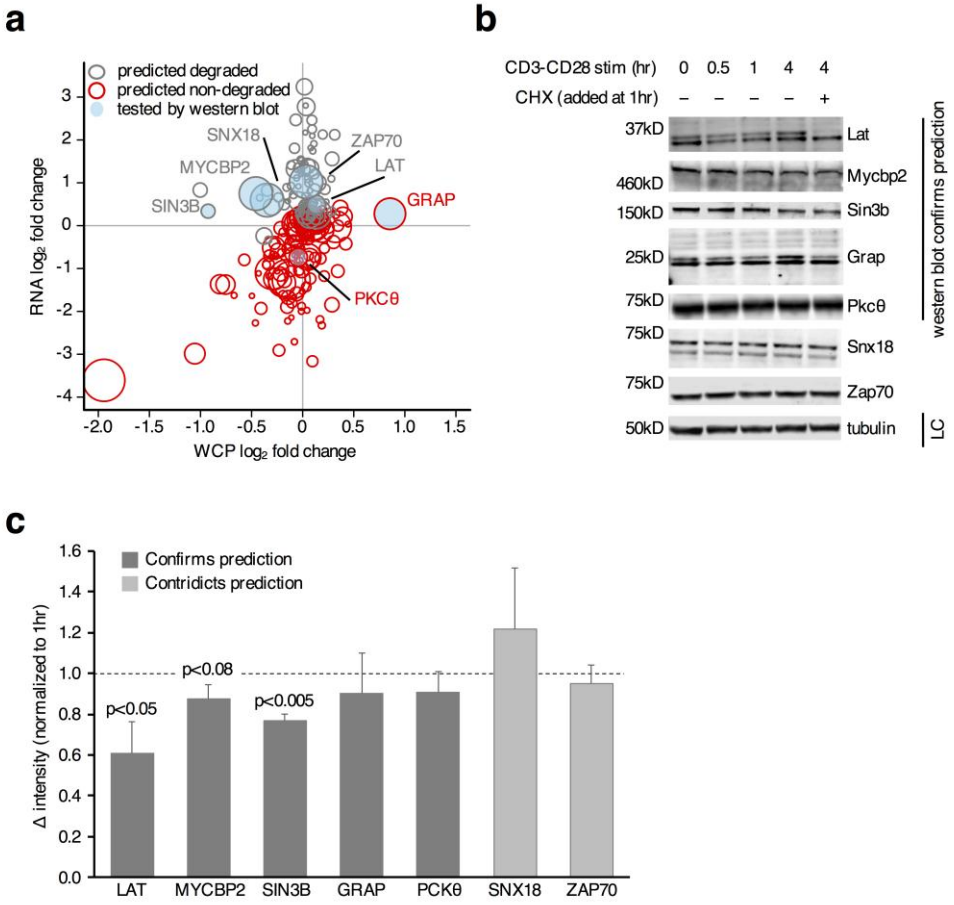


Supplementary Figure 4

RNA and protein abundance exhibit low correlation.

a, Log_2 fold change in WCP protein abundance and RNA transcript abundance identified in WCP mass spectrometry and RNA-seq experiments, respectively, in $CD4^+$ T cells rested or restimulated for 4 hours with CD3+CD28 antibody coated beads (3:1 cell:bead ratio). WCP and RNA data is shown for all identified WCP proteins (grey) or significantly increased and decreased WCP proteins (red), along with the correlation coefficients for each group. Significantly increase and decreased proteins were classified by WCP log_2 fold change p-value < 0.01, as calculated by a two-tailed student's t-test. **b**, WCP protein abundance (measured by z-score on average of rested and restimulated $CD4^+$ T cells) compared to RNA transcript abundance (measured by log_{10} transformed read count average of rested and restimulated $CD4^+$ T cells) in $CD4^+$ T cells rested or restimulated, as described in (**a**). WCP and RNA data is shown for all proteins (grey) or ubiquitylated proteins (cyan), identified in di-glycine remnant mass spectrometry experiments in $CD4^+$ T cells rested or restimulated, as described in (**a**). **a,b**, Correlation coefficients were calculated using the Pearson's method.

Supplementary Figure 5



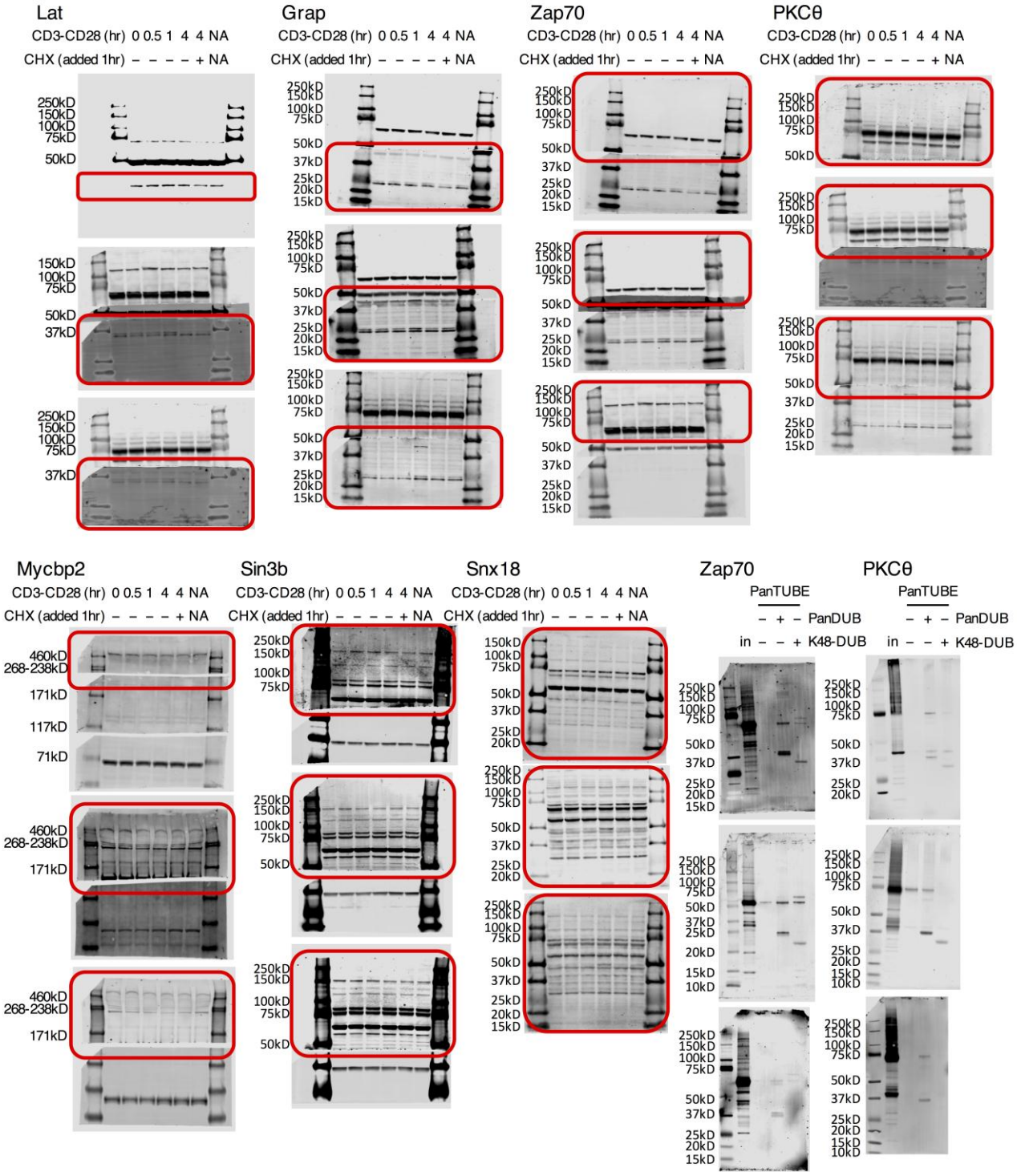
Supplementary Figure 5

Model predicting degradative or non-degradative ubiquitylation is assessed by western blot.

a, Ubiquitylation, WCP, and RNA-seq expression changes for all proteins exhibiting TCR-induced ubiquitylation (>25% increase) in CD4+ T cells unstimulated or stimulated for 4 hours with CD3+CD28 antibody coated beads (3:1 cell :bead ratio). Circle size corresponds to increase in ubiquitylation normalized $\log_2$ fold change. Proteins exhibiting consistent protein abundance and increased RNA are predicted to be degraded by ubiquitin (grey) while the remaining proteins are predicted to be non-degradative outcomes of ubiquitylation (red). Translucent blue filled circles indicate those proteins were tested for experimental validation of the prediction method. **b**, Western blot showing protein abundance of 7 selected proteins, from those described in (a), for CD4+ T cells unstimulated or stimulated with CD3+CD28 antibody (plate-bound antibody, 5μg/mL), for the indicated time-course. Cycloheximide (CHX) was added after 1 hour of stimulation CD3+CD28 antibody stimulation. Comparing protein levels at 1 hour (no CHX) to 4 hours with CHX ad ded at 1 hour suggests that LAT, MYCBP2 and SIN3B are significantly decreased, while GRAP, PKCθ, SNX18 and ZAP-70 remain stable. LC indicates loading control. Representative blots from three independent experiments are shown. **c**, Quantification of normalized intensity changes calculated for 4 hour TCR stimulation with CHX added after 1 hour compared to the protein intensity at 1hr, as described in (**b**). Decreases in protein levels with the addition of CHX (delta intensity < 1) for LAT, MYCBP2 and SIN3B indicate that these p roteins are significantly decreased in abundance. Protein band intensity was normalized to corresponding loading control intensity. Mean fold changes +/- sd of the three biological replicates are shown. Fold change of 1 indicates no change in abundance. Statistics were calculated using two-tailed, unpaired t-tests.

Supplementary Figure 6

a

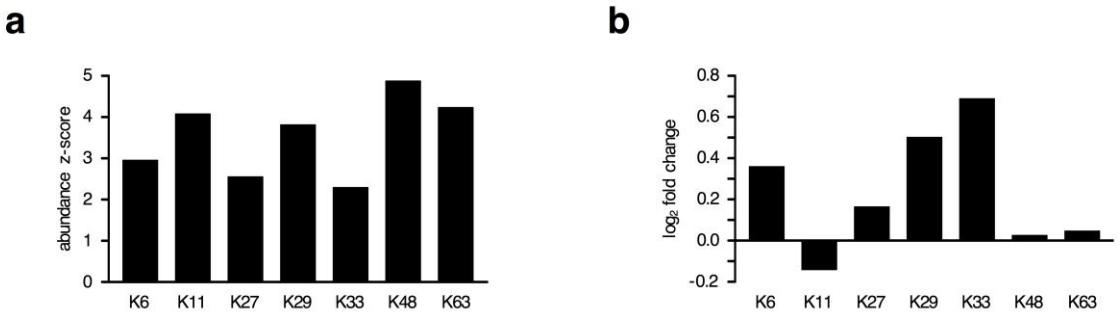


Supplementary Figure 6

Unaltered images of immunoblots.

a, Images of the uncropped and unaltered blots obtained from CD4+ T cell protein abundance experiments described in Supplementary Fig. 6 and CD4+ T cell panTUBE experiments described in Fig. 5d. For cases in which multiple blots appear in one image, the relevant blot showing the analyzed antibody is indicated by a red box.

Supplementary Figure 7



Supplementary Figure 7

Ubiquitin peptide abundance in di-glycine remnant proteome after 1 hour of CD4+ T cell stimulation.

a, Relative abundance of ubiquitin lysine peptides identified in the ubiquitin proteome of CD4+ T cells stimulated with CD3+CD 28 antibody coated beads (3:1 cell:bead ratio) for 1 hr. Relative abundance is represented by the z-score of the peptide abundance quantified by normalized intensity values from a single di-glycine immunoprecipitation experiment. **b**, Change in abundance of ubiquitin lysine peptides identified in the ubiquitin proteome of CD4+ T cells stimulated as described in (**a**). Log_2 fold change ratios are determined from a single di-glycine immunoprecipitation mass spectrometry experiment.