**Supplemental Information**

**Supplemental Tables**

**Supplemental Table 1: Complete list of sequence and phosphorylation site assignments of all identified phosphopeptides with corresponding SIC peak areas and statistics, protein accession numbers from NCBI nr, uniprot and HPRD, and gene ontology and KEGG functional annotation.** Included in this table are confident MSMS peptide assignments at >20 MOWSE score, <2 ppm mass error and logistic spectra score filter to achieve a final estimated 1% FDR by decoy database approach. Only forward database hits are included in this table.

**Supplemental Table 2: Complete list of phosphopeptides detected from every replicate and timepoint of TCR stimulation.** Included in these tables are all phosphotyrosine containing peptides with MOWSE score > 20 and mass error <2 ppm including decoy database reversed sequence hits. Reversed hits, if any for each replicate and time point are labeled with protein descriptor ###REV### and a "R" designation in database direction. Listed are the assigned names of the corresponding proteins, the position of the phosphorylation site within the protein sequences, and the assigned peptide sequence. For the peptide sequence, * represents phosphorylation, ^ or % represent assignment of heavy SILAC Arg or Lys, and # represents Met oxidation. Every reported peptide includes the Logistic Spectral Validation score and Mascot MOWSE score reflecting confidence in the sequence assignment and the Ascor, which reports the confidence in the localization of the phosphorylation site. Also reported is the mass error in ppm, the isolated mass of the peptide, the charge state, and the scan number**.**

**Supplemental Table 3: Summary of the number of phosphopeptides and phosphotyrosine sites identified from each LCMS run and the percentage of peptide SIC peak areas calculated from MSMS retention times.**

**Supplemental Table 4: Pairwise replicate correlation for evaluation of reproducibility of SIC peak areas generated from MSMS retention times or retention time alignment.**

**Supplemental Figure Legends**

**Figure S1: Western blot analysis for SLP-76 protein and specific phosphotyrosine site.** A) Expression level of SLP-76 in mutant cells and reconstituted cells. The SLP-76 expression levels in SLP-76 Y3F (J14-2D1) and SLP-76 WT (J14-76-11) cells were tested by the 9 anti-Flag western blots (Sigma-Aldrich, Monoclonal ANTI-FLAG M2 antibody produced in mouse, F3165). GAPDH was used as the loading control. There was no significant difference in the GAPDH normalized SLP-76 protein level ($p=0.6417$) in J14-76-11 and J14-2D1. B) ERK1/2 phosphorylation across a time course of TCR stimulation. Cell lysates from a time course of TCR stimulation were separated by SDS-PAGE and immunodetected with phospho-ERK1/2 and ERK1/2 specific antibodies. C) Phosphorylation of PLCγ1 across a time course of TCR stimulation. Cell lysates were separated by SDS-PAGE and immunodetected with phospho-PLCγ1 (Tyr783) and PLCγ1 specific antibodies.

**Figure S2: Pairwise replicate comparison of selected ion chromatogram peak areas**

**calculated using either MSMS retention times or retention time alignment**. Each point on the dot plots represent the SIC peak area calculated from a single phosphopeptide in two different replicate analyses for the 0 min J14-76-11 data using either MSMS based retention times exclusively ("MSMS") or retention time alignment exclusively ("RT"). Supplemental Table 3 contains an exhaustive list of pairwise correlation coefficients for all time points and both cell lines.

**Figure S3**: **Comparison of selected ion chromatogram peak areas calculated using spectral alignment retention times("Aligned RT") or MSMS retention times ("MSMS RT").** Each point on the dot plot represents a single phosphopeptide that was quantified for a single timepoint and single cell line using either MSMS based retention times or retention time alignment. In the case where more than one replicate employed the same method for calculation of the peak area, the average value is plotted.

**Figure S4: Annotated MSMS spectra for all PTM containing peptides.**

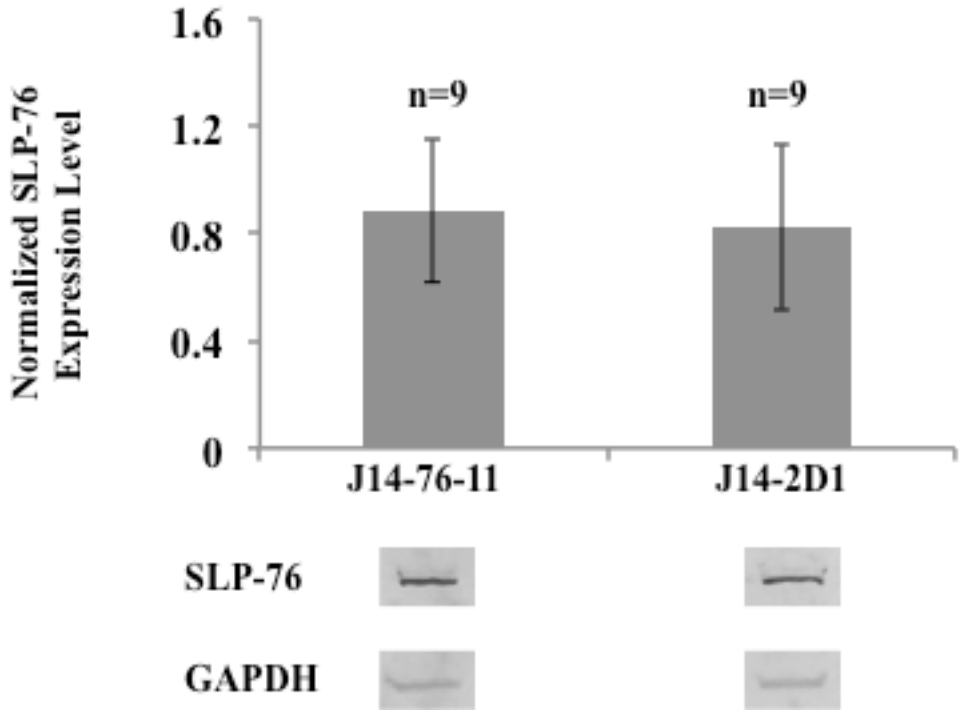
**Figure S5**: **Comparison of phosphopeptide abundance through a time course of T cell receptor stimulation of PAG, PLCγ1 and PLCγ2 in J14-76-11 and J14-2D1.** Phosphorylation kinetics of **A)** PAG, **B)** PLCγ1, and **C)** PLCγ2 in J14-76-11 (WT) and Y3F mutant J14-2D1 (Y3F) are plotted for 8 time points. Results represent means of five replicate experiments (error bars indicate standard error). "*" represents time points with significant changes ($Q$ value < 0.05) in phosphorylation abundance between WT and Y3F mutant cells.

**Figure S6**: **Comparison of phosphopeptide abundance through a time course of T cell receptor stimulation of Lck, ZAP70 and Itk in J14-76-11 and J14-2D1.** Phosphorylation kinetics of A) Lck, B) ZAP70, and C) Itk in J14-76-11 (WT) and Y3F mutant J14-2D1 (Y3F) are plotted for 8 time points. Results represent means of five replicate experiments (error bars indicate standard error). "*" represents time points with significant changes ($Q$ value < 0.05) in phosphorylation abundance between WT and Y3F cells.

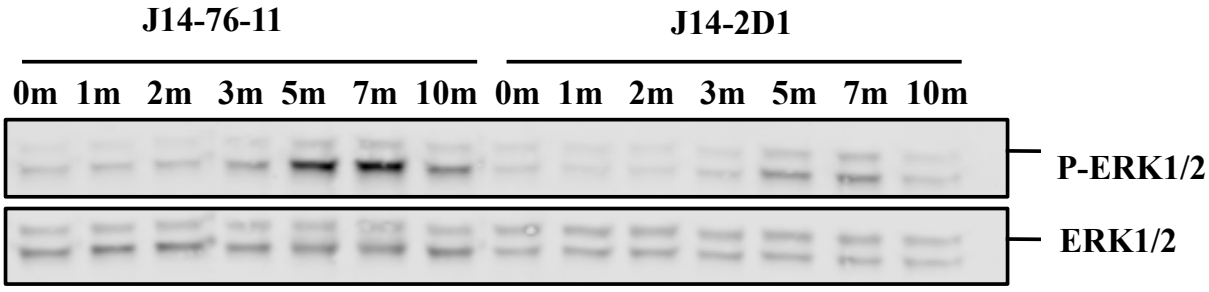
**Figure S7**: **Comparison of phosphopeptide abundance through a time course of T cell receptor stimulation of CD3 ITAMs in J14-76-11 and J14-2D1.** Phosphorylation kinetics of CD3 ITAMs in J14-76-11 (WT) and Y3F mutant J14-2D1 (Y3F) are plotted for 8 time points. Results represent means of five replicate experiments (error bars 3 indicate standard error). "*" represents time points with significant changes ($Q$ value < 0.05) in phosphorylation change between WT and Y3F mutant cells.

**Figure S1: Western blot analysis for SLP-76 protein and specific phosphotyrosine site.**

**A**




**B**




**C**

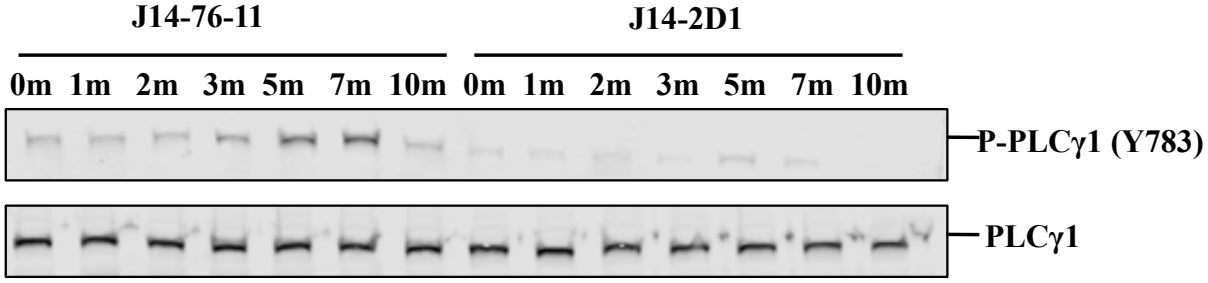


**Figure S2: Pairwise replicate comparison of selected ion chromatogram peak areas calculated using either MSMS retention times or retention time alignment**.

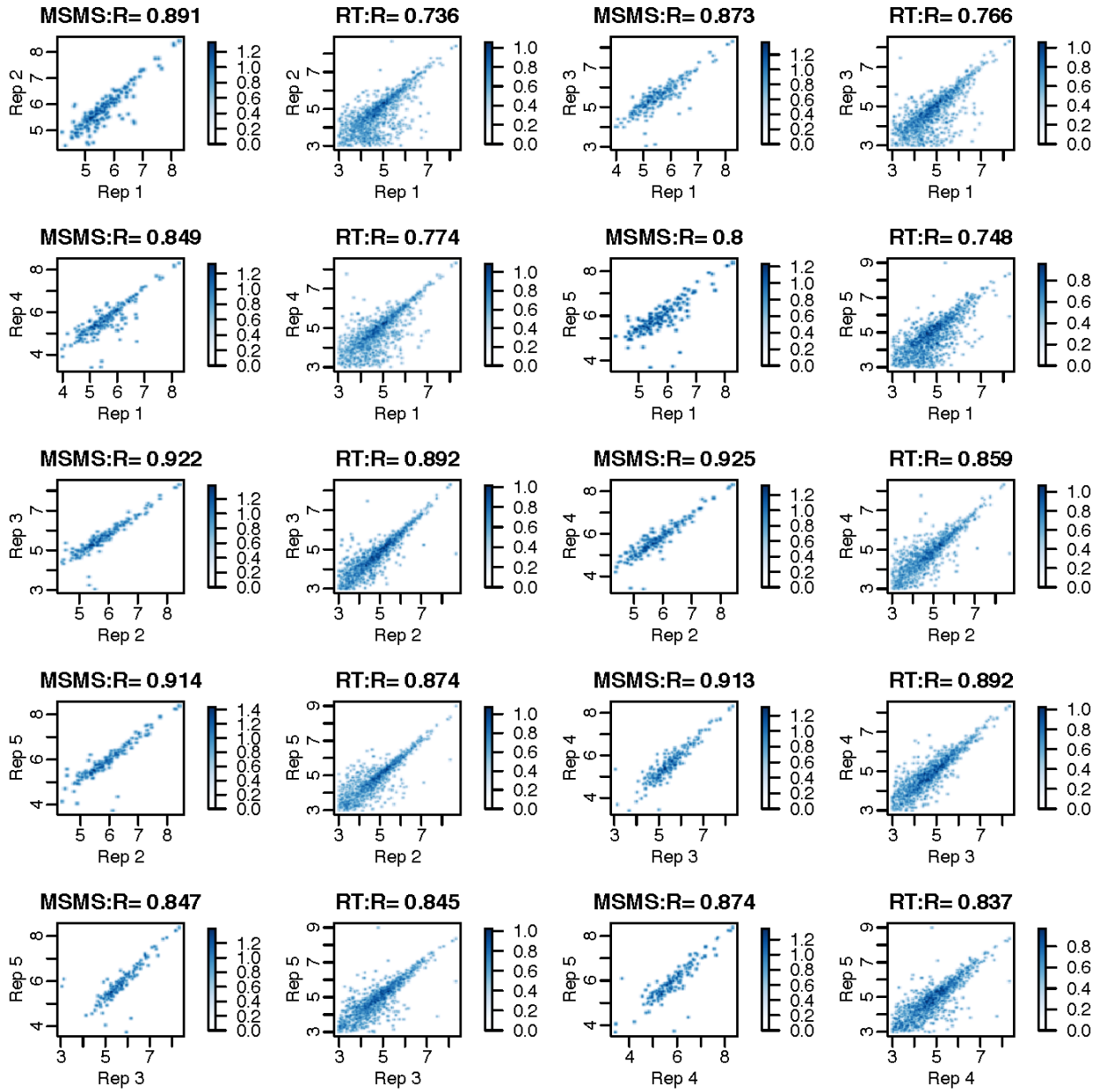


**Figure S3: Comparison of selected ion chromatogram peak areas calculated using spectral alignment retention times ("Aligned RT") or MSMS retention times ("MSMS RT").**

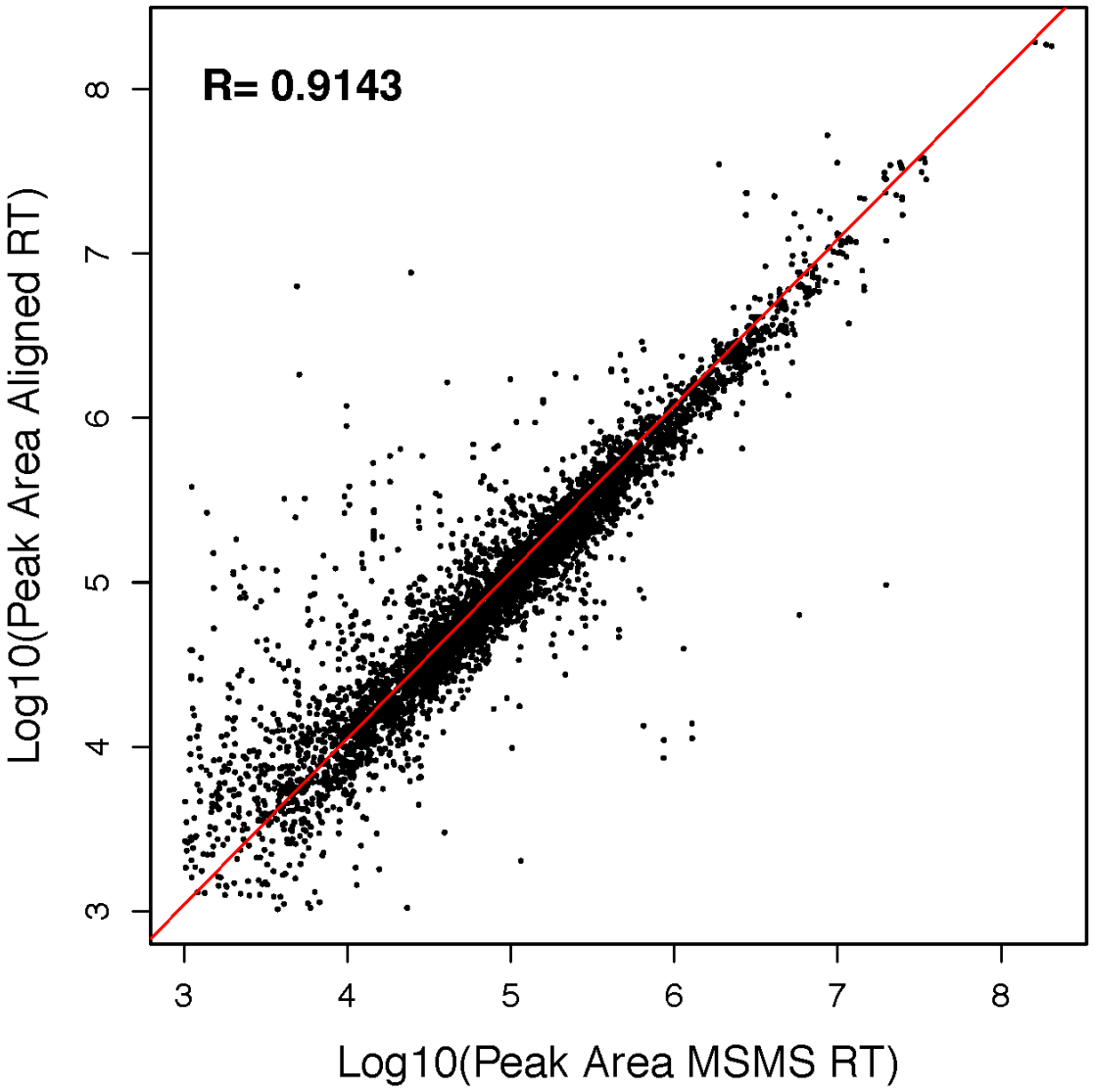
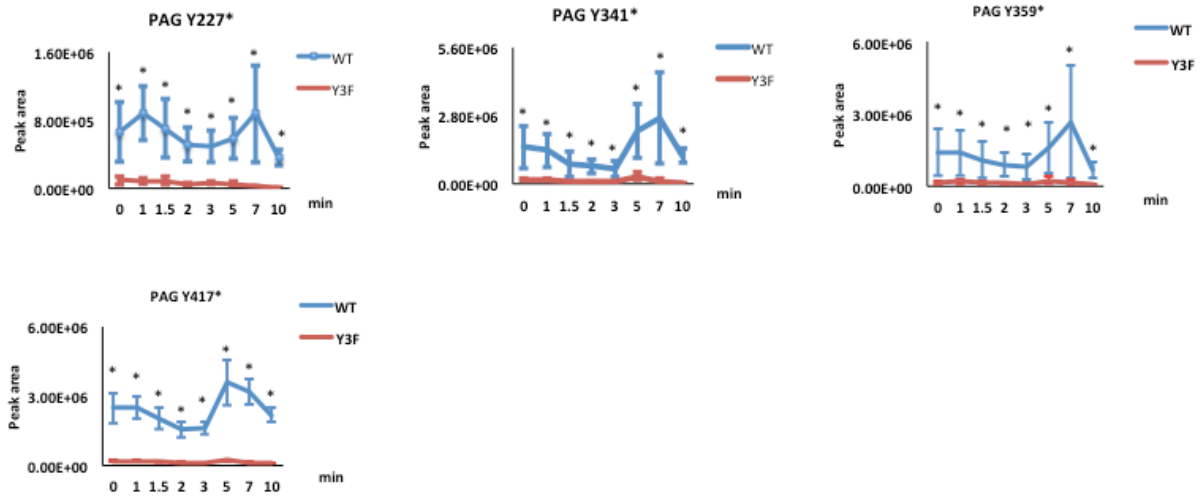


**Figure S5: Comparison of phosphopeptide abundance through a time course of T cell receptor stimulation of PAG, PLCγ1 and PLCγ2 in J14-76-11 and J14-2D1.**

**A**

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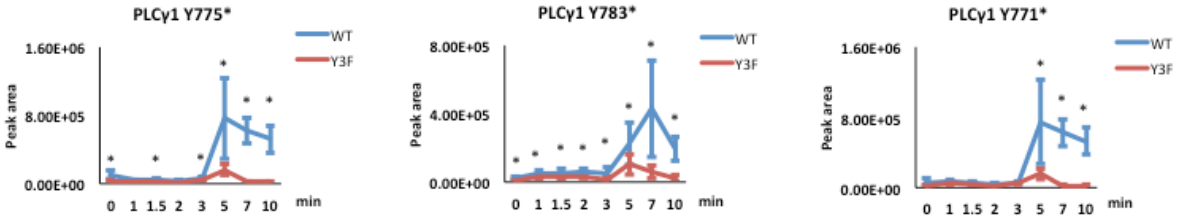



**C**

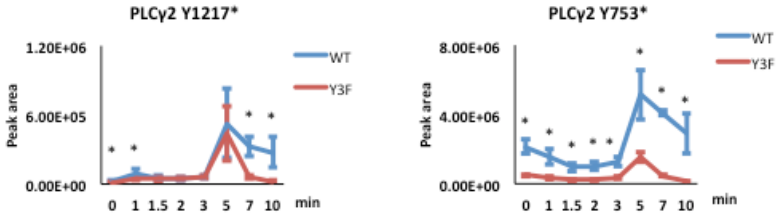
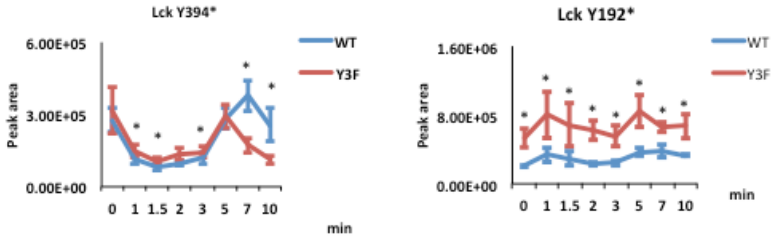


**Figure S6**: **Comparison of phosphopeptide abundance through a time course of T cell receptor stimulation of Lck, ZAP70 and Itk in J14-76-11 and J14-2D1.**

**A**

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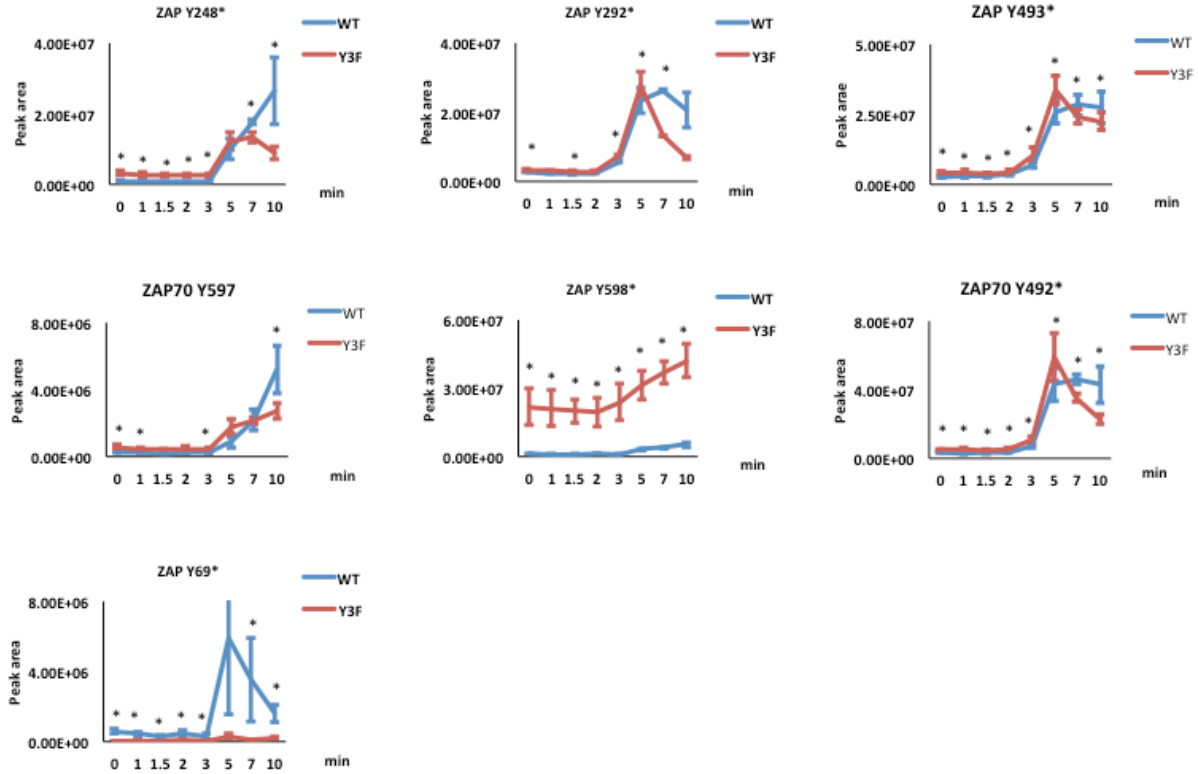


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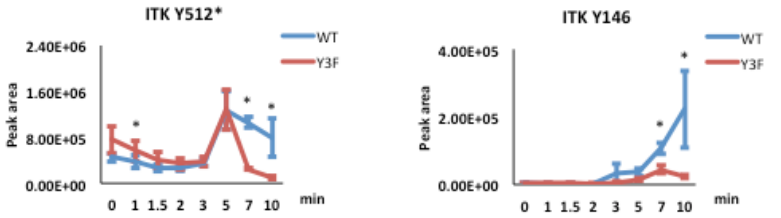

**Figure S7: Comparison of phosphopeptide abundance through a time course of T cell receptor stimulation of CD3 ITAMs in J14-76-11 and J14-2D1.**