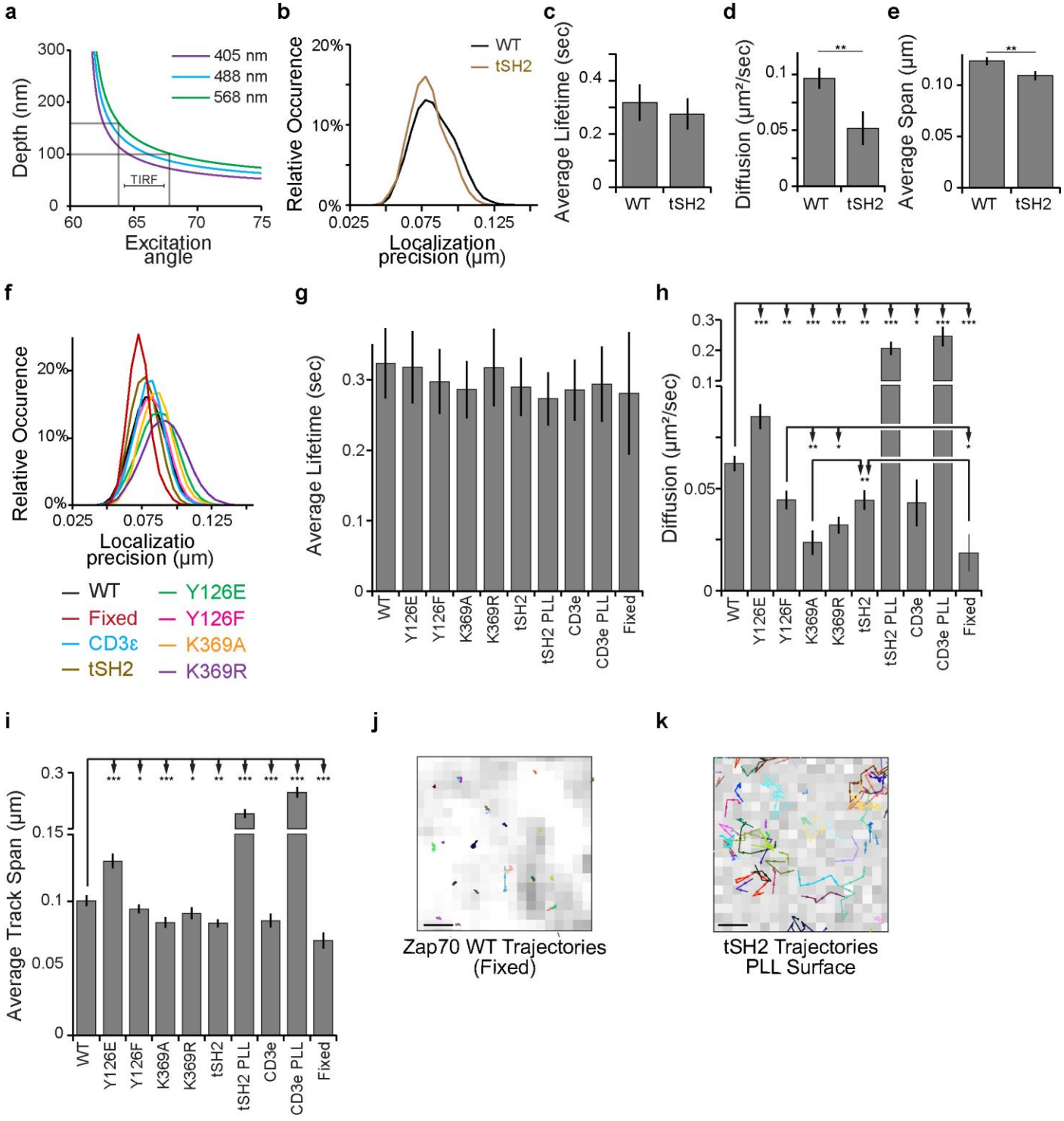
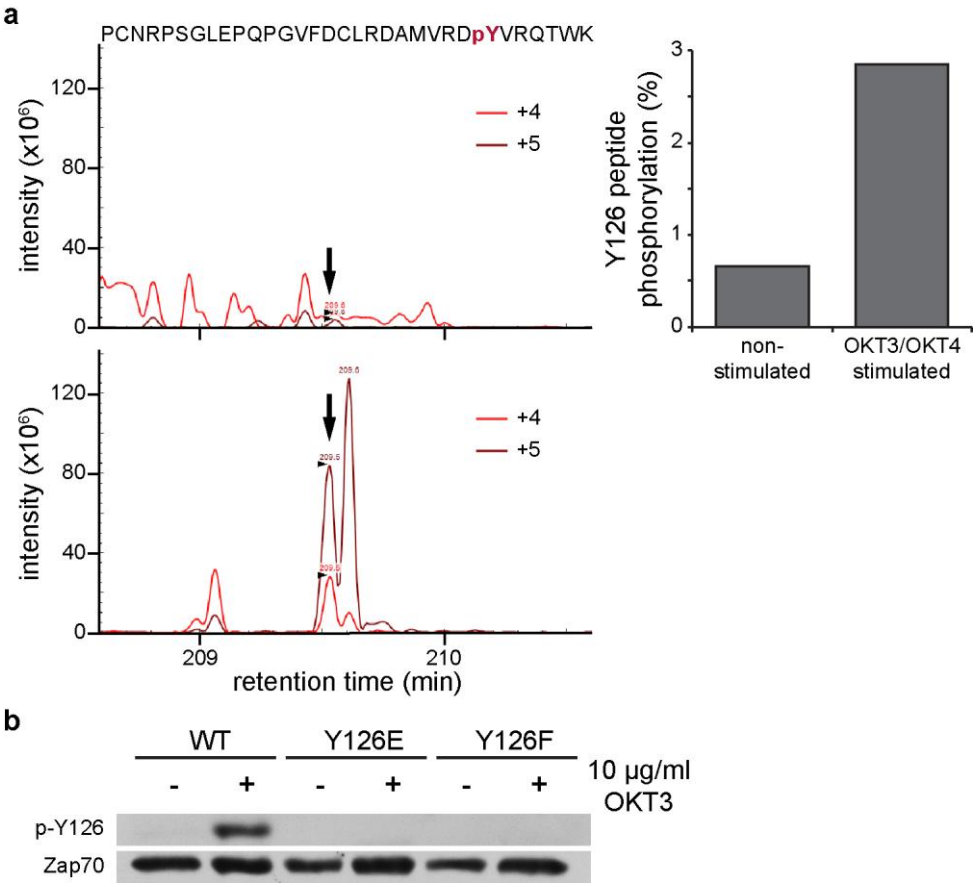

a
b
c
d
e
Depth (nm)
300
200
100
0
405 nm
488 nm
568 nm
TIRF
60
65
70
75
Excitation angle
Relative Occurence
20%
10%
0%
WT
tSH2
0.025
0.075
0.125
Localization precision (μm)
Average Lifetime (sec)
0.4
0.2
0
WT
tSH2
Diffusion (μm²/sec)
0.1
0.05
**
Average Span (μm)
0.10
f
g
h
Localizatio
WT
Fixed
CD3ε
tSH2
Y126E
Y126F
K369A
K369R
tSH2 PLL
CD3e
CD3e PLL
i
j
k
Average Track Span (μm)
0.15
Zap70 WT Trajectories (Fixed)
tSH2 Trajectories PLL Surface

Supplementary Figure 1

SPT analyses of Zap70 at the T cell plasma membrane.

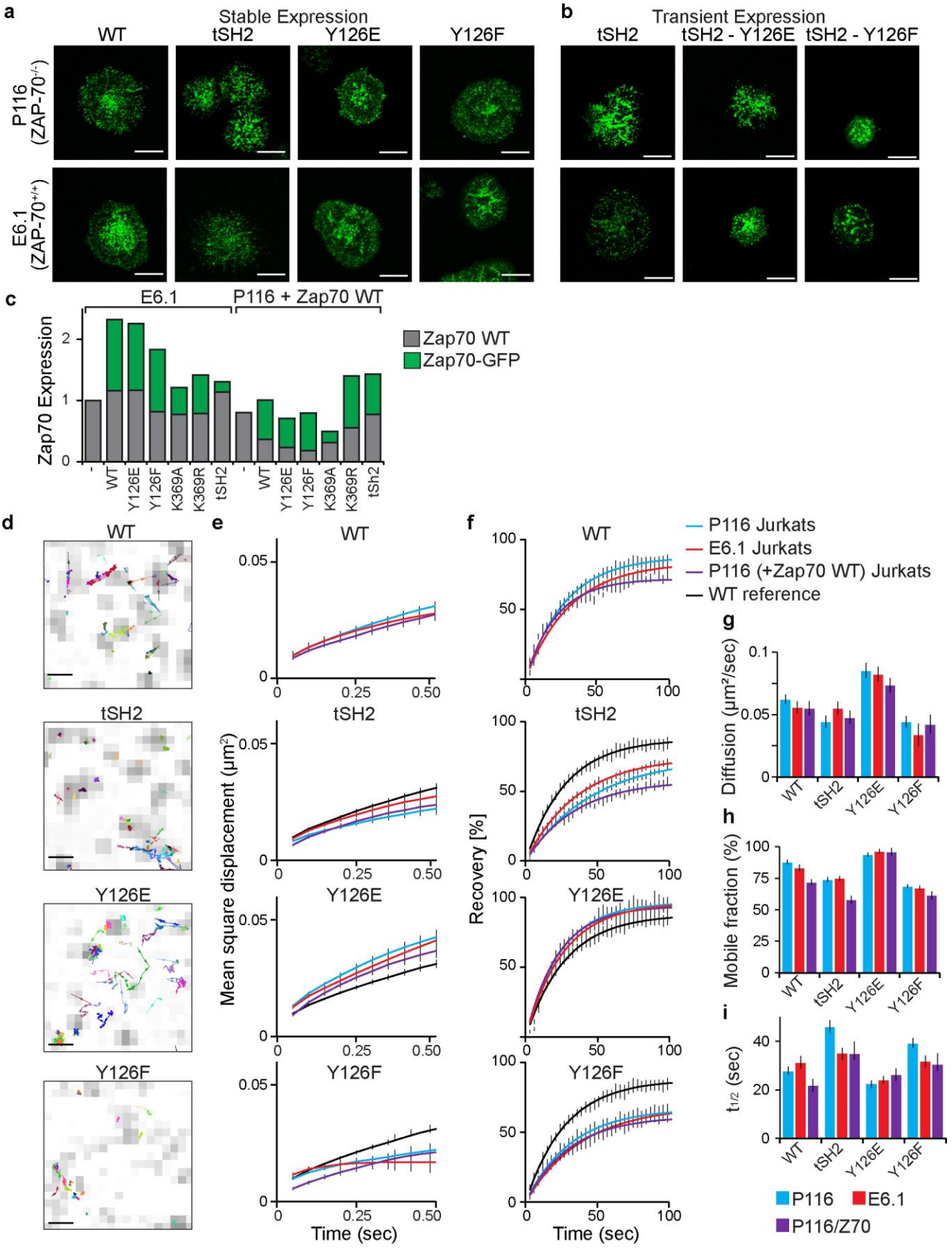
(**a**) Total internal reflection fluorescent (TIRF) excitation at 64-68 degrees limits single molecule detection to 100-150 nm above the glass surface. (**b**) Distribution of single molecule localization precisions from all trajectories of murine wild-type Zap70 (WT; black) and the tandem SH2 module (tSH2; brown) fused to PATagRFP in primary 5c.c7 T cells. (**c**) Average Trajectory lifetimes. (**d**) The average diffusion rate (τ = 500 ms). (**e**) Average trajectory spans (distance between the two furthest points in a trajectory). (**f**) Distribution of single molecule localization precisions from all trajectories in P116 Jurkat T cell lines expressing different forms of human Zap70 fused to PATagRFP. (**g**) Average trajectory lifetimes. (**h**) The average diffusion rate (τ = 500 ms). (**i**) Average trajectory spans. The T cell receptor (specifically CD3ε) was tracked on poly-L-lysine surfaces to reference free movement within the plasma membrane. (**j**) Zap70 trajectories in a fixed cell expressing WT overlaid with diffraction limited TCR microclusters (gray pixels). (**k**) Representative tracks of Zap70's tSH2 module on non-stimulatory PLL surface. Data represents at least 3 independent experiments and 18 different cells for each condition. Significance was assessed with a two-tailed unpaired *t*-test between indicated samples (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).



Supplementary Figure 2

Inducible phosphorylation of Tyr126 in Zap70.

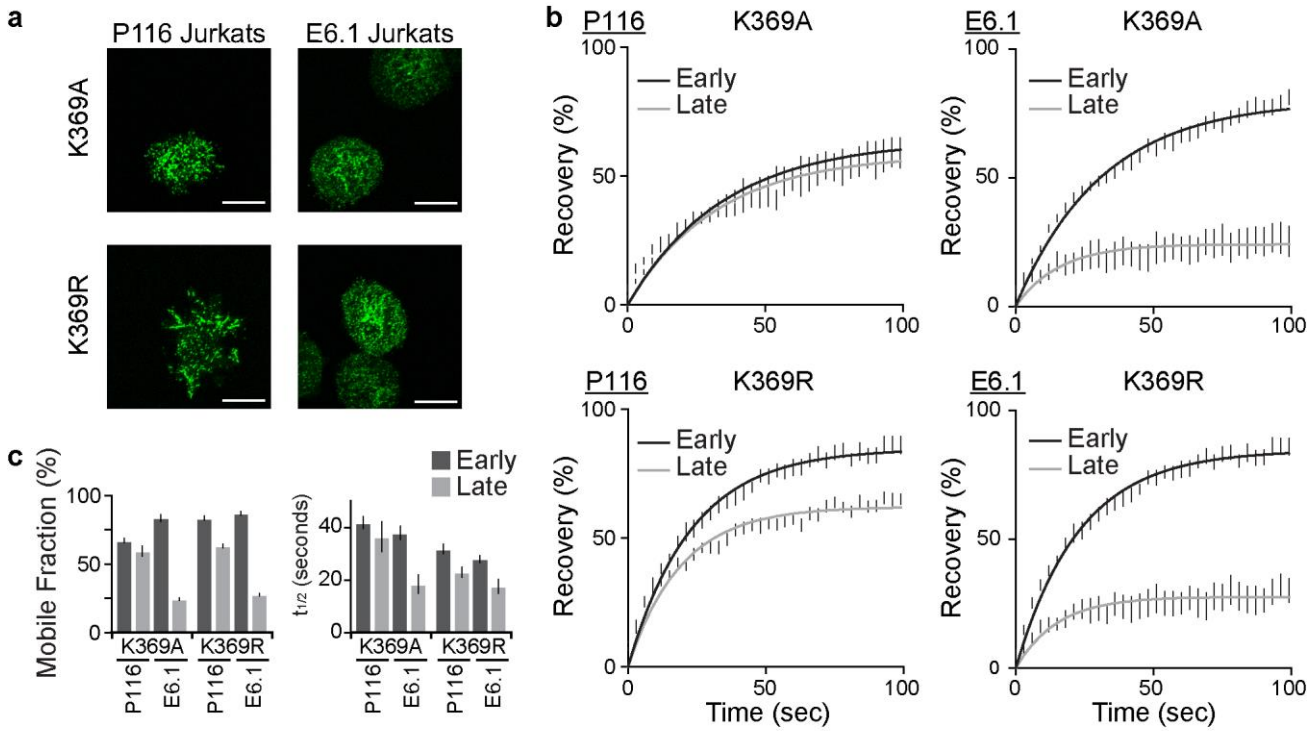
(**a**) Detection of Y126 phosphorylation upon OKT3/OKT4 stimulation by mass spectrometry. Chromatogram of p-Y126 containing peptide (sequence shown above) for non-stimulated (top) and stimulated (bottom) T cells. Graph (right) shows the percentage of p-Y126 phosphorylated peptide. (**b**) Western Blot analyses of lysates from non- and OKT3-stimulated P116 Jurkat T cells expressing WT, Y126E and Y126F using a rabbit serum raised against a peptide containing p-Y126.


a
Stable Expression
WT
tSH2
Y126E
Y126F
P116 (ZAP-70-/-)
E6.1 (ZAP-70+/+)
b
Transient Expression
tSH2
tSH2 - Y126E
tSH2 - Y126F
c
E6.1
P116 + Zap70 WT
Zap70 Expression
Zap70 WT
Zap70-GFP
-
WT
Y126E
Y126F
K369A
K369R
tSH2
d
WT
tSH2
Y126E
Y126F
e
Mean square displacement (µm²)
Time (sec)
f
Recovery [%]
Time (sec)
P116 Jurkats
E6.1 Jurkats
P116 (+Zap70 WT) Jurkats
WT reference
g
Diffusion (µm²/sec)
h
Mobile fraction (%)
i
t1/2 (sec)
P116
E6.1
P116/Z70

Supplementary Figure 3

Effects of endogenous Zap70 on the mobility of Zap70 mutants.

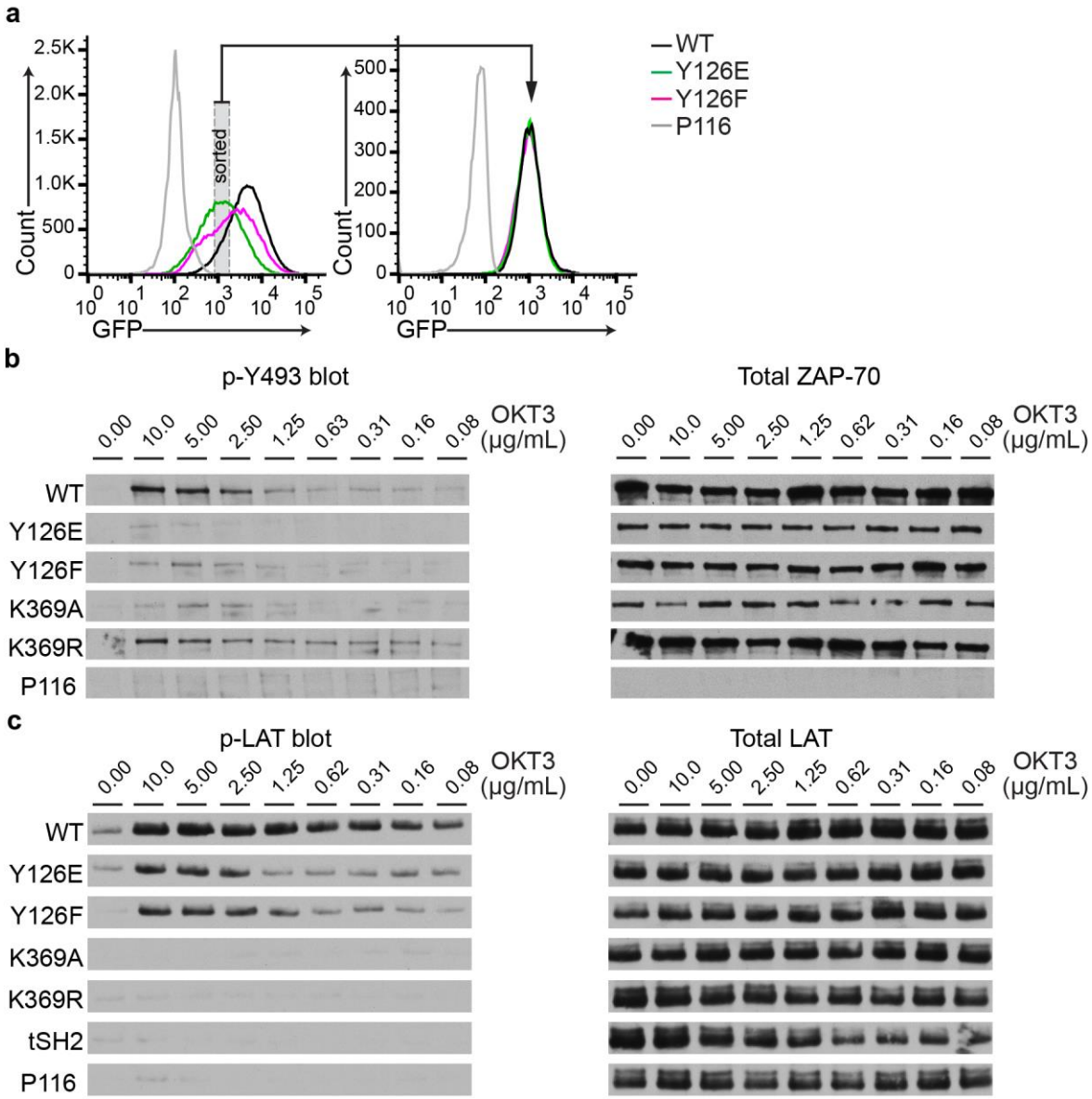
(**a**) Confocal images of microclusters from fluorescence recovery after photobleaching (FRAP) experiments of GFP tagged Zap70 variants stably expressed in P116 (top) and E6.1 (bottom) Jurkat T cells. Scale bars represents 10 μm. (**b**) Microclusters of GFP tagged tSH2, tSH2-Y126E and tSH2-Y126F transiently expressed in P116 (top) and E6.1 (bottom) Jurkat T cells. (**c**) Relative expression of Zap70-GFP constructs stably expressed in E6.1 cells with endogenous Zap70 and P116 exogenously expressing wild-type Zap70. (**d**) Trajectories of WT, tSH2, Y126E and Y126F fused to PATagRFP in E6.1 Jurkat T cells expressing endogenous Zap70. Trajectories are overlaid on diffraction-limited images of TCR microclusters (CD3ζ-GFP; gray; 500nm scale bar). Pixels are 160×160 nm. (**e**) Comparison of mean square displacement of Zap70 variants in P116 (cyan), E6.1 (red), and P116 + wild-type Zap70 Jurkat T cells (purple), with P116 WT curve as reference (black solid line). (**f**) FRAP recovery curves for WT, tSH2, Y126E and Y126F fused to GFP in P116 (cyan), E6.1 Jurkat T cells (red), and P116 + wild-type Zap70 Jurkat T cells (purple). The data for WT in P116 Jurkat T cells is inserted as reference in each plot (solid black curve). (**g**) The average diffusion rate (τ = 500 ms) from SPT. (**h**) Bar graphs of average mobile fraction from FRAP. (**i**) average recovery rates ($t_{1/2}$) from FRAP. Tracking data represents averages from at least ten cells from 3 independent experiments with ± s.e.m. Pixels are 160×160 nm. FRAP data represent averages with s.e.m. from at least 11 different cells from 3 or more independent experiments. Error bars for mobile fractions and recovery rates ($t_{1/2)}$ represent the 95% confidence interval.



Supplementary Figure 4

Inhibition of Zap70's release at late time points requires Zap70's kinase activity.

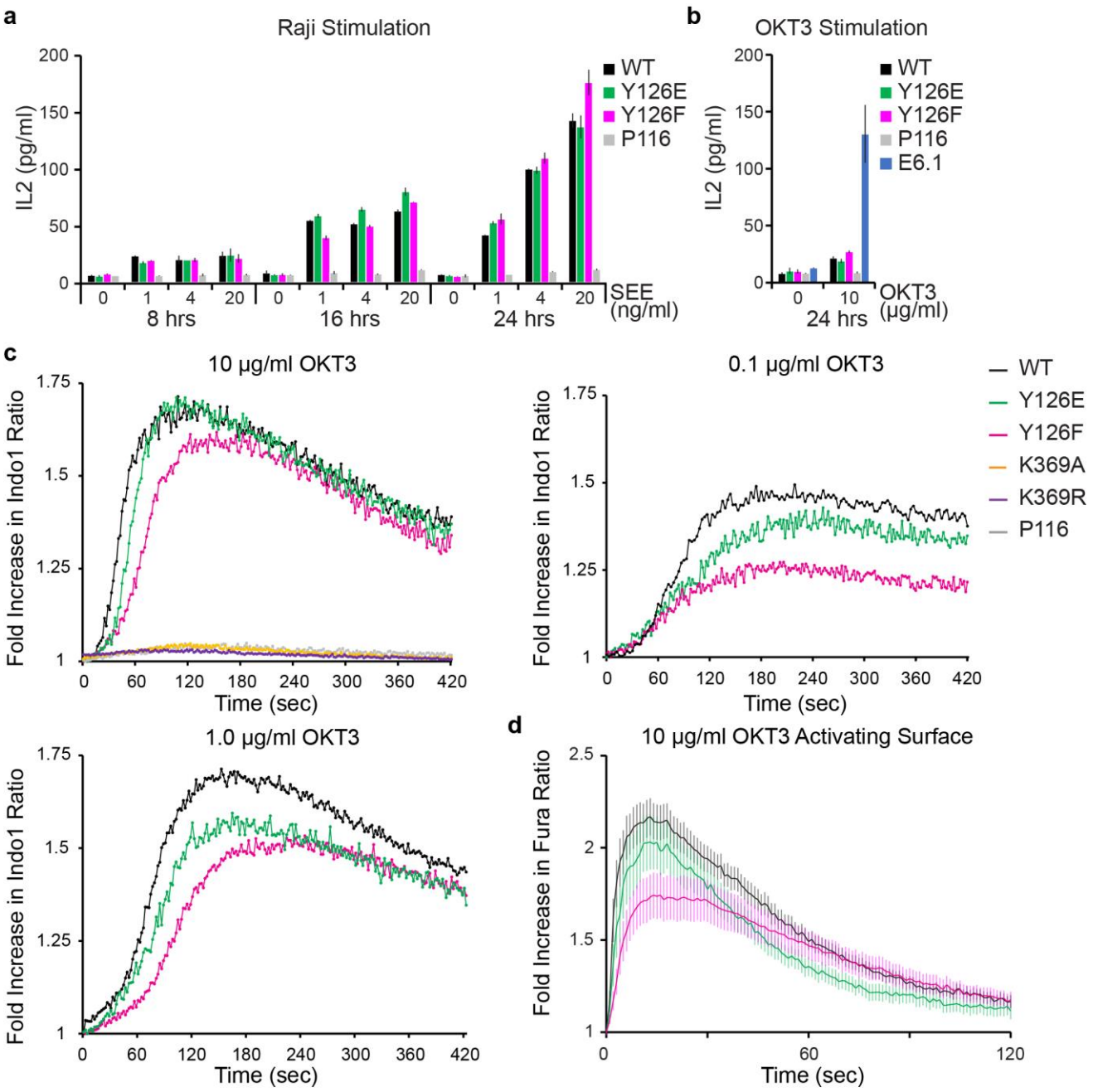
(**a**) Confocal images of microclusters from fluorescence recovery after photobleaching (FRAP) experiments of GFP tagged Zap70 ATP binding mutants (specifically K369A and K369R) stably expressed in P116 (left) and E6.1 (right) Jurkat T cells. (**b**) Kinase dead Zap70s were analyzed in Zap70 deficient P116 (left) and E6.1 Jurkat T cells (right). Early time points (<10 min; black lines) are compared to data at late time points (>30 min; gray lines). (**c**) Bar graphs of average mobile fraction and average recovery rates ($t_{1/2}$). FRAP data represent averages with s.e.m. from at least 15 different cells from 3 or more independent experiments. Error bars for mobile fractions and recovery rates $t_{1/2}$ represent the 95% confidence interval.



Supplementary Figure 5

Zap70's release from the TCR controls downstream signaling.

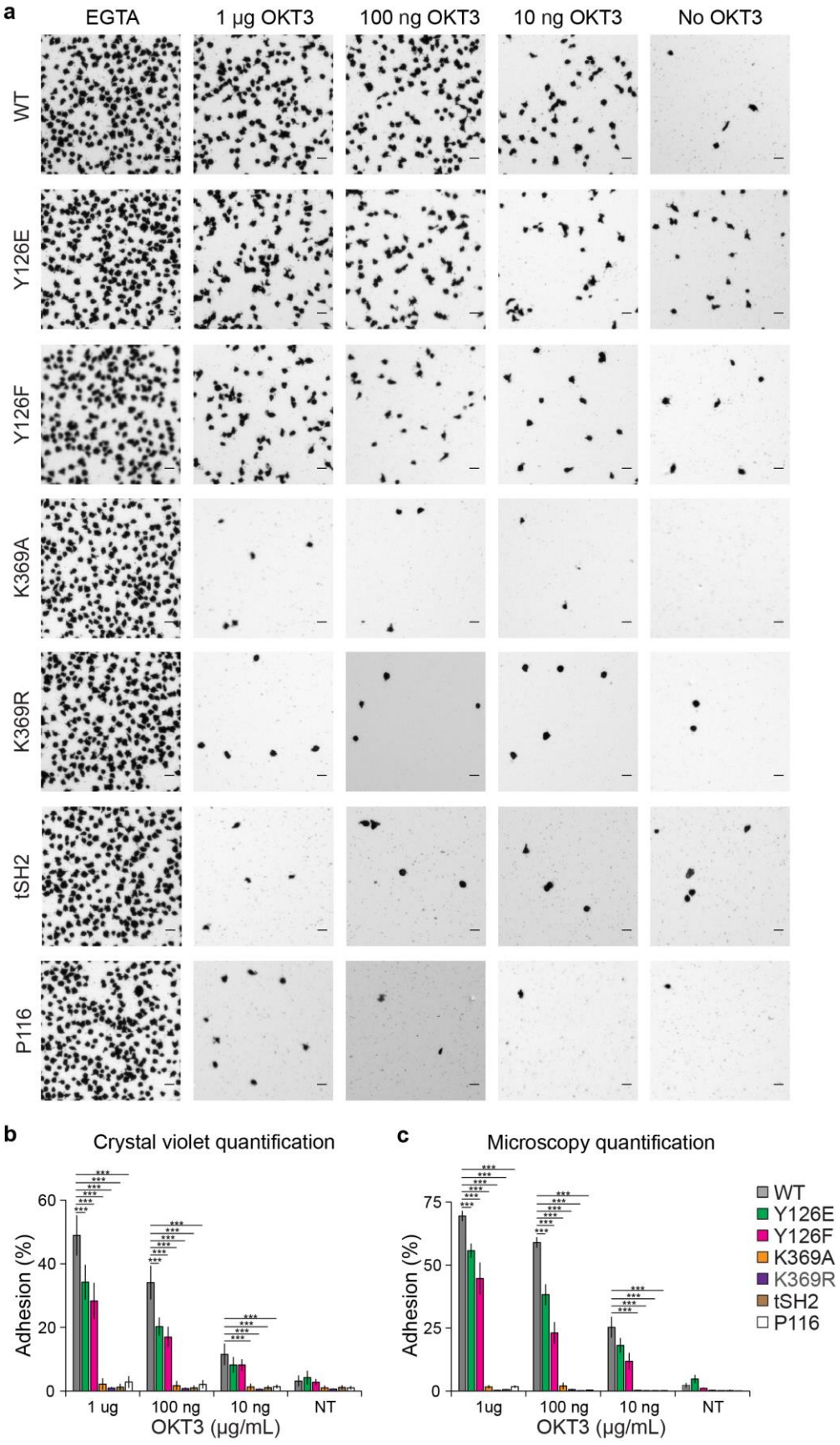
(**a**) Flow cytometry analysis of original (left) and expression matched (right) WT, Y126E and Y126F expressing P116 Jurkat T cell lines. Original cell lines were sorted for overlapping expression level (gray bar) and cultivated for one week before re-analyses. (**b**) Western-blot analyses of Y493 phosphorylation (p-Y493, left) and total Zap70 protein (right) in non-sorted P116 Jurkat T cell lines after stimulation with different concentrations of OKT3. (**c**) Western-blot analyses of Lat phosphorylation (p-Y171 + p-Y191; left) and total Lat protein (right) in P116 Jurkat T cell lines after stimulation with different concentrations of OKT3.



Supplementary Figure 6

IL-2 secretion and Ca^{2+} flux of Jurkat T cell lines.

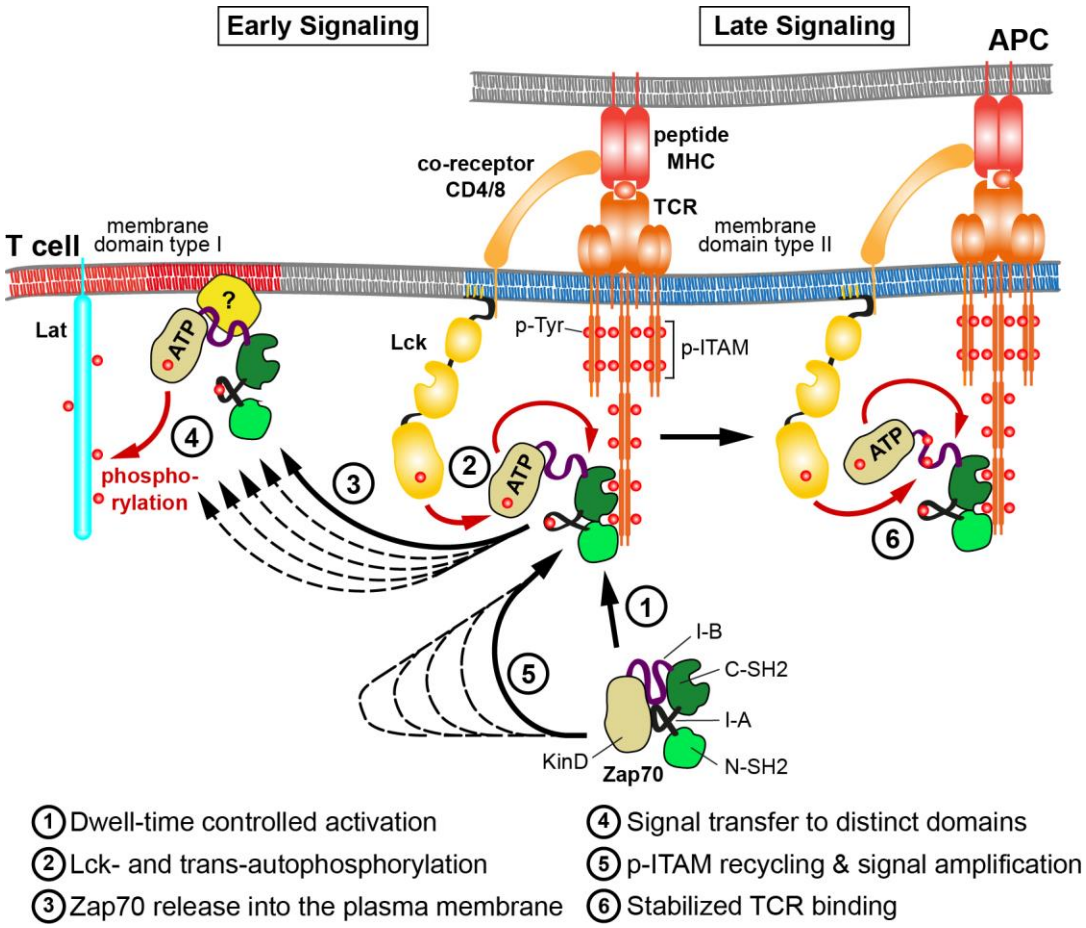
(**a**) IL-2 secretion of P116 Jurkat T cell lines after stimulation with SEE pulsed Raji B cells. (**b**) IL-2 secretion of WT, Y126E and Y126F expressing P116 Jurkat T cell lines and E6.1 Jurkat T cells after stimulation with OKT3. Data represents averages ± s.e.m. from at least two independent measurements with duplicates. (**c**) Calcium flux of P116 Jurkat T cell lines stably expressing WT, Y126E and Y126F with 10 µg/ml, 1 µg/ml and 0.1 µg/ml OKT3. Ca-Flux was analyzed for T cells with matched GFP expression. The P116 Zap70 null cells and Kinase dead Zap70 mutants (K369A and K369R) are shown for 10 µg/ml OKT3 stimulation. Curves are population averages of the violet/blue ratio of Indo1 ratiometric calcium dye. (**d**) ER calcium release of sorted P116 WT, Y126E, and Y126F cell lines assessed by ratiometric Fura2 imaging on activating surfaces (10 µg/ml OKT3) in the absence of extracellular calcium. Curves represent averages ± s.e.m. from at least three independent experiments with at least ten cells analyzed from each experiment.


a
EGTA
1 µg OKT3
100 ng OKT3
10 ng OKT3
No OKT3
WT
Y126E
Y126F
K369A
K369R
tSH2
P116
b
Crystal violet quantification
Adhesion (%)
60
40
20
0
1 ug
100 ng
10 ng
NT
OKT3 (µg/mL)
c
Microscopy quantification
Adhesion (%)
75
50
25
0
1ug
100 ng
10 ng
NT
OKT3 (µg/mL)
WT
Y126E
Y126F
K369A
K369R
tSH2
P116

Supplementary Figure 7

Alteration of Zap70's release from the TCR reduces adhesion to ICAM-1 after T cell stimulation.

(**a**) Representative images of P116 Jurkat T cells stably expressing WT and mutant Zap70s adhered to ICAM-1 surfaces after stimulation with different concentrations of OKT3 (indicated above). Left column shows maximal adhesion upon treatment with $MgCl_2$/EGTA. (**b**) Quantification of cell adhesion by extraction of crystal violet from adherent cells in relation to $MgCl_2$/EGTA treatment. (**c**) Surface area covered by P116 Jurkat T cell lines in relation to $MgCl_2$/EGTA treatment. Scale bars are 20 μm. Error bars represent s.e.m. from three independent experiments with duplicates. Three images of different regions quantified for each sample. Significance was assessed with a two-tailed unpaired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).



Supplementary Figure 8

The 'catch-and-release' model for TCR signal amplification and dispersion.

Proposed 'Catch-And-Release' model for early and late Zap70 signaling at the TCR. Signaling events are indicated by numbered circles. In brief, upon TCR engagement by pMHC, Zap70 kinase is recruited to the Lck-phosphorylated ITAMs of the TCR-CD3-complex. During early signaling Zap70 is Lck- and/or auto-transphosphorylated on Y126, activated and binds ATP. Altogether, this induces Zap70's release from the TCR into the plane of the plasma membrane. Released Zap70 translocates to other membrane domains to phosphorylate its substrates (e.g. LAT). The vacated p-ITAM is available to recruit, activate and release additional Zap70 molecules. This turns the TCR into a 'catalytic unit' that amplifies antigenic stimuli. During later stages of T cell activation Zap70 release does not take place and Zap70 maintains TCR signaling while stably associated with p-ITAMs, probably through phosphorylation of Y315 and Y319.

	5c.c7 Primary T cells		
	Lifetime (sec)	**Diffusion (μm^2/sec)**	**Span (μm)**
WT	0.318 ± 0.068	0.096 ± 0.010	0.123 ± 0.004
tSH2	0.276 ± 0.058	0.052 ± 0.015	0.109 ± 0.004

Supplementary Table 1. SPT analyses of wild-type Zap70 and Zap70's tSH2 module in Primary T cells.

SPT analyses of WT and tSH2 Zap70 fused to PAtagRFP in primary 5c.c7 T cells on stimulatory surfaces. The diffusion (μm^2/sec), span (μm), and lifetime (sec) are averages ± s.e.m from at least 18 different cells from 3 or more independent experiments.

	P116 T cells		
	Lifetime (sec)	**Diffusion (μm^2/sec)**	**Span (μm)**
WT	0.324 ± 0.050	0.062 ± 0.004	0.100 ± 0.004
Y126E	0.318 ± 0.051	0.085 ± 0.006	0.128 ± 0.005
Y126F	0.298 ± 0.046	0.044 ± 0.005	0.094 ± 0.003
K369A	0.286 ± 0.041	0.024 ± 0.006	0.084 ± 0.004
K369R	0.318 ± 0.055	0.032 ± 0.004	0.091 ± 0.004
tSH2	0.290 ± 0.041	0.044 ± 0.005	0.083 ± 0.003
tSH2 on PLL	0.273 ± 0.038	0.206 ± 0.021	0.190 ± 0.011
CD3ε	0.286 ± 0.043	0.043 ± 0.011	0.085 ± 0.005
CD3ε on PLL	0.294 ± 0.054	0.245 ± 0.032	0.247 ± 0.014
Fixed	0.281 ± 0.087	0.019 ± 0.009	0.071 ± 0.006

Supplementary Table 2. SPT analyses of wild-type and mutant Zap70s in P116 cells.

SPT analyses of WT and mutant Zap70s fused to PAtagRFP stably expressed in P116 T cells. T cells bound to non-activating (on PLL) and stimulatory surfaces (OKT3). The lifetime (sec), span (μm), and diffusion (μm^2/sec) are averages ± s.e.m from at least 18 different cells from 3 or more independent experiments.

	P116 T cells			E6.1 T cells			P116 + wild-type Zap70 T cells		
	Mobile fraction (f)	**$t_{1/2}$ (sec)**	**R^2**	**Mobile fraction (f)**	**$t_{1/2}$ (sec)**	**R^2**	**Mobile fraction (f)**	**$t_{1/2}$ (sec)**	**R^2**
WT	85 ± 2%	22.7 ± 1.6	0.99	81 ± 3%	25.8 ± 2.9	0.97	72 ± 2%	21.8 ± 2.6	0.95
tSH2	70 ± 2%	37.9 ± 2.5	0.99	72 ± 2%	30.1 ± 2.6	0.99	58 ± 3%	35.0 ± 4.9	0.96
Y126E	94 ± 2%	19.5 ± 1.3	0.99	92 ± 2%	18.2 ± 1.5	0.98	96 ± 3%	26.3 ± 2.5	0.98
Y126F	66 ± 2%	32.4 ± 2.1	0.99	65 ± 2%	26.3 ± 2.5	0.97	62 ± 3%	30.6 ± 4.4	0.95
K369A	64 ± 2%	34.8 ± 2.9	0.98	80 ± 3%	31.4 ± 3.1	0.98	67 ± 2%	22.3 ± 2.4	0.96
K369R	80 ± 1%	26.1 ± 1.3	0.99	85 ± 2%	23.1 ± 1.9	0.99	73 ± 2%	24.3 ± 1.3	0.97
CD3ζ	13 ± 1%	15.3 ± 4.4	0.68	21 ± 1%	12.3 ± 2.3	0.87	-	-	-

Supplementary Table 3. FRAP analyses of wild-type and mutant Zap70s.

FRAP analyses of wild-type and mutant Zap70s stably expressed in P116 (left), E6.1 (middle), and wild-type Zap70 P116 Jurkat T cells on OKT3 surfaces. The recovery ($t_{1/2}$) is a measure for the time it takes 50% of the mobile fraction (f) to recover. Averages and s.e.m. were derived from exponential fits (R^2) of each recovery curve from at least 15 cells from three independent experiments.

	P116 T cells		
	Mobile fraction (f)	**$t_{1/2}$ (sec)**	**R^2**
tSH2	73 ± 2%	31.0 ± 3.0	0.98
tSH2-Y126E	80 ± 2%	18.3 ± 1.7	0.97
ZAP-70 Y126F	54 ± 2%	34.5 ± 4.1	0.97

Supplementary Table 4. FRAP analyses of wild-type and mutant tandem SH2 domain modules.

FRAP analyses of wild-type and mutant tSH2s transiently expressed in P116 (left) and E6.1 (right) Jurkat T cells on OKT3 surfaces. The recovery ($t_{1/2}$) is a measure for the time it takes 50% of the mobile fraction (f) to recover. Averages and s.e.m. were derived from exponential fits (R^2) of each recovery curve from at least 15 cells over three independent experiments.

	Diffusion (μm^2/sec)		
	P116 Cells	**E6.1 T cells**	**P116 + wild type Zap70 T cells**
WT	0.062 ± 0.004	0.056 ± 0.005	0.055 ± 0.006
Y126E	0.085 ± 0.006	0.082 ± 0.006	0.073 ± 0.006
Y126F	0.044 ± 0.005	0.034 ± 0.009	0.042 ± 0.007
K369A	0.024 ± 0.006	0.059 ± 0.007	0.062 ± 0.005
K369R	0.032 ± 0.004	0.059 ± 0.006	0.051 ± 0.005
tSH2	0.044 ± 0.005	0.055 ± 0.005	0.048 ± 0.005

Supplementary Table 5. SPT analyses of wild-type and mutant Zap70s in P116 cells compared to E6.1 and Zap70 re-expression P116 cells.

SPT analyses of WT and mutant Zap70s fused to PAtagRFP stably expressed in P116 (left), E6.1 (middle), and Zap70 WT re-expression P116 Jurkat T cells on OKT3 surfaces. The diffusion (μm^2/sec) averages ± s.e.m are from at least 11 different cells from 3 or more independent experiments.

	Early				Late			
	Diffusion (μm^2/sec)	**Mobile fraction (f)**	**$t_{1/2}$ (sec)**	**R^2**	**Diffusion (μm^2/sec)**	**Mobile fraction (f)**	**$t_{1/2}$ (sec)**	**R^2**
WT	0.062 ± 0.004	85 ± 2%	22.7 ± 1.6	0.99	0.026 ± 0.012	30 ± 1%	21.8 ± 2.5	0.95
Y126E	0.085 ± 0.006	94 ± 2%	19.5 ± 1.3	0.99	0.040 ± 0.016	22 ± 1%	11.7 ± 2.4	0.80

Supplementary Table 6. Mobility of Zap70 WT and Y126E at early and late signaling.

SPT analyses measured the diffusion (μm^2/sec) of WT and Y126E fused to PATagRFP stably expressed in P116 Jurkat T cells on OKT3 surfaces at early (<10 minutes) and late (>30 minutes) signaling. FRAP analyses of WT and Y126E fused to GFP and stably expressed in P116 Jurkat T cells on OKT3 surfaces. The recovery ($t_{1/2}$) is a measure for the time it takes 50% of the mobile fraction (f) to recover. Averages and s.e.m. were derived from exponential fits (R^2) of each recovery curve from at least 10 cells over three independent experiments.

	Cell line	Early			Late		
		Mobile fraction (f)	$t_{1/2}$ (sec)	R^2	Mobile fraction (f)	$t_{1/2}$ (sec)	R^2
K369A	P116	64 ± 2%	34.8 ± 2.9	0.98	59 ± 4%	36.4 ± 5.7	0.95
K369A	E6.1	80 ± 3%	31.4 ± 3.1	0.98	24 ± 1%	18.5 ± 3.4	0.86
K369R	P116	80 ± 1%	26.1 ± 1.3	0.99	63 ± 2%	22.9 ± 2.0	0.98
K369R	E6.1	85 ± 2%	23.1 ± 1.9	0.99	28 ± 1%	17.6 ± 2.7	0.92

Supplementary Table 7. FRAP of Zap70 kinase mutants at early and late signaling in P116 and E6.1 cells.

FRAP analyses of Zap70 kinase mutants stably expressed in P116 and E6.1 Jurkat T cells on OKT3 surfaces at early (<10 minutes) and late (>30 minutes) signaling. The recovery ($t_{1/2}$) is a measure for the time it takes 50% of the mobile fraction (f) to recover. Averages and s.e.m. were derived from exponential fits (R^2) of each recovery curve from at least 15 cells over three independent experiments.

	ZAP-70 D461N : Competitor			
Competitor	**1 : 3**	**1 : 1**	**1 : 0.3**	**ATP**
WT	0.22 ± 0.01	0.50 ± 0.01	0.75 ± 0.01	+
ZAP-70 Y126E	0.69 ± 0.05	0.87 ± 0.04	0.97 ± 0.05	+
ZAP-70 Y126F	0.17 ± 0.03	0.39 ± 0.04	0.64 ± 0.04	+
tSH2	0.13 ± 0.02	0.23 ± 0.03	0.38 ± 0.09	+
tSH2 Y126E	0.41 ± 0.03	0.68 ± 0.08	0.80 ± 0.15	+
tSH2 Y126F	0.14 ± 0.30	0.23 ± 0.05	0.40 ± 0.13	+
ZAP-70 D461N	0.23 ± 0.01	0.46 ± 0.01	0.75 ± 0.02	+
ZAP-70 K369A	0.06 ± 0.02	0.12 ± 0.03	0.26 ± 0.04	+
ZAP-70 K369R	0.26 ± 0.01	0.48 ± 0.01	0.70 ± 0.08	+
ZAP-70 D461N	0.25 ± 0.05	0.49 ± 0.05	0.74 ± 0.04	-
ZAP-70 K369A	0.22 ± 0.06	0.41 ± 0.07	0.67 ± 0.03	-
ZAP-70 K369R	0.32 ± 0.02	0.62 ± 0.01	0.84 ± 0.01	-

Supplementary Table 8. Competition of wild-type and mutant Zap70s for p-CD3γ binding.
The table represents binding (%) of tagged kinase dead Zap70 (D461N) to p-CD3γ when competing with the indicated ratios of untagged wild-type and mutant Zap70s. 100% equals binding of D461N without competitor. Each value represents averages of triplicate samples from at least three independent experiments and errors represent s.e.m.

	K_D (nM)	k_{on} (1/Ms)	k_{off} (1/s)	X^2	R^2
WT	20.1 ± 0.4	4.0 ± 0.9E+04	6.9 ± 0.9E-04	0.22 ± 0.08	>0.99
tSH2 Y126E*	76.8 ± 12.2	2.6 ± 0.5E+04	27.0 ± 4.7E-04	0.01 ± 0.01	>0.99
tSH2 Y126F	22.4 ± 0.2	4.1 ± 0.5E+04	9.0 ± 0.1E-04	0.08 ± 0.05	>0.99

Supplementary Table 9. Binding constants of wild-type and mutant tandem SH2 domain modules binding to p-CD3γ.

Binding constants (K_D), on-rates (k_{on}), and off-rates (k_{off}) for each tSH2 variant from three independent experiments. tSH2-Y126E* fits best to a two-component model and the effective binding constants shown here are the averages weighted by contributions of a weak and strong binding constants. Errors represent standard error about the mean. X^2 and R^2 values represent the average of fits from all experiments.

	ATP	1st set of constants				2nd set of constants				Fit X^2	Fit R^2
		K_D^1 (nM)	k_{on}^1 (1/Ms) 1x10^4	k_{off}^1 (1/s) 1x10^{-4}	Pop^1	K_D^2(nM)	k_{on}^2 (1/Ms) 1x10^5	k_{off}^2 (1/s) 1x10^{-2}	Pop^2		
WT	-	10.0±4.1	2.5±0.8	2.1±0.2	92±2%	114±12.8	1.3±0.1	1.5±0.2	8±2%	0.06±0.03	>0.99
WT	+	22.5±6.7	4.1±0.7	8.5±0.9	72±2%	116±15.4	1.6±0.1	1.9±0.2	28±8%	0.05±0.02	>0.99
K369A	-	3.6±0.8	3.5±0.7	1.1±0.1	89±2%	87.1±4.3	1.7±0.1	1.4±0.1	11±2%	0.09±0.03	>0.99
K369A	+	5.1±0.9	2.0±0.3	1.0±0.2	93±1%	88.7±10.6	1.8±0.2	1.5±0.1	7±1%	0.03±0.01	>0.99
K369R	-	3.6±0.9	5.0±0.9	1.3±0.1	85±3%	90.2±17.2	2.3±0.4	1.6±0.1	15±3%	0.13±0.05	>0.99
K369R	+	11.9±1.7	10.6±1.5	11.7±0.7	46±1%	70.6±15.2	2.8±0.3	2.3±0.1	54±1%	0.13±0.03	>0.99
D461N	-	7.1±1.0	7.2±0.7	5.1±0.7	71±1%	101±13.6	1.3±0.2	1.3±0.1	29±1%	0.24±0.05	>0.99
D461N	+	11.2±1.0	10.3±1.1	11.3±0.9	49±1%	86.2±5.3	2.4±0.2	2.1±0.2	51±1%	0.16±0.04	>0.99

Supplementary Table 10. Binding constants of wild-type and kinase dead Zap70s binding to p-CD3γ derived from a two-component fit.

Binding constants (K_D^{1+2}), on-rates (k_{on}^{1+2}), and off-rates (k_{off}^{1+2}) for wild-type and kinase dead Zap70 were derived from a two-component fit representing high affinity and the low affinity states. Binding constants represent means and standard errors about the mean from at least three independent experiments. X^2 and R^2 values represent the average of fits from all experiments.

	ATP	K_D^{eff} (nM)	k_{on}^{eff}(1/Ms) x 10^4	k_{off}^{eff} (1/s) x 10^{-3}
WT	-	18.0 ± 2.5	3.3 ± 1.0	1.4 ± 0.20
WT	+	49.1 ± 11.3	7.5 ± 0.4	5.9 ± 1.00
K369A	-	12.7 ± 0.1	4.9 ± 0.9	1.6 ± 0.20
K369A	+	11.0 ± 1.7	3.1 ± 0.3	1.1 ± 0.10
K369R	-	16.5 ± 4.6	7.4 ± 1.4	2.4 ± 0.03
K369R	+	53.6 ± 7.9	19.8 ± 2.5	11.3 ± 0.80
D461N	-	33.8 ± 5.0	8.8 ± 0.7	4.0 ± 0.10
D461N	+	48.8 ± 3.9	17.1 ± 0.5	10.8 ± 0.90

Supplementary Table 11. Effective binding constants of wild-type and kinase dead Zap70s binding to p-CD3γ.

Effective binding constants (K_D^{eff}), on-rates (k_{on}^{eff}), and off-rates (k_{off}^{eff}) for wild-type and kinase dead Zap70 were derived from the relative contribution of the high affinity and the low affinity state. Binding constants represent means and standard errors about the mean from at least three independent experiments. X^2 and R^2 values represent the average of fits from all experiments.

Antibody	Clone	Catalog #	Company
Mouse anti CD3epsilon	OKT3	16-0037	eBiosciences
Mouse anti Myc-tag	9E10	MA1-980	Fisher Scientific
Mouse anti HA-tag	12CA5	610090	Becton Dickinson
Mouse anti-GFP mAb (biotinylated)	9F9	600-306-215	Rockland
Mouse anti Zap70 C-terminus	29	610240	BD Biosciences
Mouse anti Zap70 N-terminus	2F3.2	05-253	Upstate
Rabbit anti Zap70	ER227(2)	2869-1	Epitomics
Rabbit anti phosphoY292 Zap70	-	sc-12945	Santa Cruz
Rabbit anti phosphoY319 Zap70	-	sc-12946	Santa Cruz
Rabbit anti phosphoY492 Zap70	EP2291Y	2215-1	Epitomics
Mouse anti phosphoY493 Zap70	717917	MAB7694	R&D Systems
Rabbit anti phosphoY126 Zap70	-	-	Generated with ProSci, Inc.
Mouse anti phosphoTyrosine	4G10	BE0194	Bio-X-Cell
Rabbit anti phosphoY191 Lat	-	3584	Cell Signaling Technology
Rabbit anti phosphoY171 Lat	-	3581S	Cell Signaling Technology
Rabbit anti Lat	-	06-807	Millipore

Supplementary Table 12. List of primary antibodies.