Encoding optical control in LCK kinase to quantitatively investigate its activity in live cells

Ardiyanto Liaunardy-Jopeace1, Ben L Murton1, Mohan Mahesh2, Jason W Chin2© & John R James1©

LCK is a tyrosine kinase that is essential for initiating T-cell antigen receptor (TCR) signaling. A complete understanding of LCK function is constrained by a paucity of methods to quantitatively study its function within live cells. To address this limitation, we generated LCK*, in which a key active-site lysine is replaced by a photocaged equivalent, using genetic code expansion. This strategy enabled fine temporal and spatial control over kinase activity, thus allowing us to quantify phosphorylation kinetics in situ using biochemical and imaging approaches. We find that autophosphorylation of the LCK active-site loop is indispensable for its catalytic activity and that LCK can stimulate its own activation by adopting a more open conformation, which can be modulated by point mutations. We then show that CD4 and CD8, T-cell coreceptors, can enhance LCK activity, thereby helping to explain their effect in physiological TCR signaling. Our approach also provides general insights into SRC-family kinase dynamics.

Biological systems rely on enzymes such as kinases to transmit information between the nodes of cell-signaling networks, often to transduce extracellular ligand-binding events into intracellular information. An important example of this is found in T cells, an essential cell type of our adaptive immune system that can discriminate between healthy cells and those that are infected by pathogens. Expression of the TCR complex at the cell surface allows the T cell to probe potentially infected host cells by scrutinizing their surface for expression of peptide fragments of pathogens presented within the MHC protein (pMHC). Upon TCR binding to cognate pMHC, a cascade of intracellular signaling is initiated from the TCR that leads to the T cell either directly killing the infected cells or instructing other cell-types to do so.

The most proximal event following pMHC binding is the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the intracellular tails of the TCR by LCK, a prototypic member of the SRC-family tyrosine kinases (SFK) that is almost exclusively expressed in T cells. The phosphorylated ITAMs then recruit proteins with SRC-homology 2 (SH2) domains, such as ZAP70, a cytoplasmic tyrosine kinase. Bound ZAP70 is phosphorylated by LCK, primarily at tyrosine 319 (Y319), which leads to its activation and subsequent phosphorylation of downstream effector molecules that drive multiplex signaling pathways. LCK kinase activity is therefore crucial in translating the TCR-pMHC interaction into intracellular signals in T cells. Understanding how the kinase activity of LCK is controlled within T cells at the molecular level is important not only for our fundamental understanding of TCR signal transduction but for suggesting new means by which its activity could be modulated therapeutically, given the deleterious effect of T-cell-mediated autoimmunity and its aberrant regulation in certain leukemias.

Previous studies have shown that the SH2 domain of LCK can bind intramolecularly to a phosphorylated residue (Y505) at the C terminus to adopt a closed autoinhibitory conformation, which is a general feature of the SFK regulatory mechanism. Phosphorylation of Y505 is catalyzed by C-terminal SRC kinase (CSK) and antagonized primarily by the membrane-bound tyrosine phosphatase CD45. This modification can regulate the conformations that LCK can adopt, affecting its activity. Full activation of LCK also requires phosphorylation at Y394 in the activation loop of the kinase domain. In addition, LCK can be bound by the T-cell coreceptors CD4 and CD8, transmembrane proteins that can both bind to the MHC protein and engage with LCK through a Zn2+ ‘clasp’. The functional effect of the coreceptors on T-cell signaling has been extensively studied during thymocyte development, but it remains unclear whether they have a direct influence on LCK kinase activity.

Current in vitro methods used to investigate how LCK, or indeed any SFK, functions at the molecular level invariably depend on assaying its kinase activity after removal from the cellular environment. Experiments are invariably performed in solution on nonphysiological substrates that are unlikely to faithfully replicate kinase function when normally constrained to the plasma membrane. Authors of a recent study did address this latter issue by tethering LCK to lipid vesicles, but this accomplishment required altering the N-terminal structure of the kinase to anchor it to the bilayer.

Conversely, most in vivo studies of LCK function have been limited by the inability to initiate kinase activity directly and so normally rely on steady-state measures of catalytic activity that do not provide the quantitative detail required for a mechanistic understanding. Recent methods have been designed to address this limitation, principally by inserting chemically- or optically-controlled domains into kinases to...
allogsterically modulate activity\textsuperscript{20–22}. This approach has found some success, although not with LCK, but all of these methods require extensive alterations to the native kinase structure that could interfere sterically with potential protein interactions and may not represent the ‘true’ kinase in \textit{situ}.

Encoding new functionality at specific sites of a protein when expressed in cells through the use of genetically encoded unnatural amino acid incorporation\textsuperscript{23,24} has enabled the \textit{in vivo} control of protein function with a precision more commonly associated with \textit{in vitro} approaches\textsuperscript{25}. By combining this approach with a cellular reconstitution of proximal TCR triggering with defined components\textsuperscript{26}, we have developed a direct and quantitative method to assay the kinase activity of native LCK within the cellular environment. Engineering LCK so that its catalytic activity can be spatiotemporally controlled within live cells by brief light stimulation provides a new platform for ‘\textit{in vivo} biochemistry’, which we use to show that LCK is critically dependent on Y394 phosphorylation in its active-site loop for its kinase activity and that the T-cell coreceptors can enhance LCK function at limiting kinase levels.

**RESULTS**

**Engineering a photocaged LCK kinase using unnatural amino acid incorporation**

To quantitatively investigate LCK kinase function when localized in the plasma membrane of live cells, we needed precise temporal control over the initiation of its enzymatic activity. We noted that lysine at position 273 (K273) in the active site of human LCK is essential for its enzymatic activity, where mutation of this residue disrupts ATP binding and substrate phosphate transfer\textsuperscript{27}. We anticipated that engineering control of substrate binding at this position would provide a mechanism to modulate LCK kinase activity. We used unnatural amino acid (UAA) mutagenesis to genetically incorporate a photocaged variant of lysine (pc-Lys) at K273, which can be rapidly uncaged under ultraviolet (~350–400 nm) illumination, leaving a scarless lysine residue\textsuperscript{25,28} (Fig. 1a). The pc-Lys residue can be easily accommodated in the active site of LCK without alteration to the kinase structure and is expected to sterically occlude ATP binding (Supplementary Fig. 1a).

Using the UAA system optimized for efficient incorporation in mammalian cells\textsuperscript{25,28}, we expressed human LCK fused to eGFP with

\begin{figure}[ht]
\centering
\includegraphics[width=\textwidth]{Figure1}
\caption{Engineering of a photocaged LCK kinase using UAA incorporation. (a) Diagram showing photocaged lysine (pc-Lys) and the removal of its caging group (red) by UV light. (b) Flow cytometry of HEK cells transfected with ZAP70-mRuby2 and the components required for pc-Lys incorporation into LCK-eGFP (LCK*), grown in the absence or presence of pc-Lys. (c) Histogram showing percentage of transfected cells (ZAP70*) that expressed LCK*. (d) Fluorescence micrographs showing localization of LCK* and wild-type LCK-eGFP in HEK cells. Scale bar, 5 μm. (e) Western blot of LCK* using an LCK-specific antibody. The band at ~82 kDa is LCK*, and the band at ~31 kDa is truncated LCK, terminated at the internal amber stop codon. (f) Representative blot showing ZAP70 phosphorylation at Y319 using a phosphospecific antibody (green) and LCK* expression (red). HEK-TCR cells coexpressing LCK* and ZAP70 were illuminated at different intensities for the indicated period. Right-hand lane shows phosphorylation by wild-type (WT) LCK-eGFP at steady state. (g) Quantification of blots in f. Intensity of pY319 band was normalized to the corresponding intensity of the LCK* band in each lane and scaled relative to the normalized value of Y319 phosphorylation by WT LCK (right-hand lane in f). (h) Representative blot of ZAP70 Y319 phosphorylation by LCK* after photocaging at 12 mW/cm\textsuperscript{2} for 30 s. Time after initiation of uncaging when cells were flash frozen is shown. (i) Quantification of blots in h. ZAP70 Y319 phosphorylation by LCK* as a function of time following illumination. Data are scaled relative to the final time point. White dotted lines in blots indicate sections from the same blot presented at different brightness for visual clarity. Data in g and i were fit using a three-parameter logistic function, presented as mean ± s.e.m. from independent experiments (n = 3 in g; n = 4 in i). Uncropped blots are shown in Supplementary Data Set 1. Source data for graphs are in Supplementary Data Set 2.

\end{figure}
the codon for K273 mutated to the amber stop codon (LCK$_{K273-TAG}$) in HEK-293T cells. Cells were transfectted with all of the components required for UAA incorporation, as well as ZAP70 kinase fused to the mRuby2 fluorophore. Cells transfected in normal medium showed expression of ZAP70 alone, but the addition of pc-Lys led to the coexpression of photocaged LCK (LCK*) (Fig. 1b). This finding confirmed that in the absence of pc-Lys, translation of LCK$_{K273-TAG}$ was efficiently terminated at the internal stop codon. We found that ~40% of ZAP70-expressing cells also incorporated pc-lys into LCK* (Fig. 1c), which localized correctly to the plasma membrane (Fig. 1d). Western analysis using an anti-LCK antibody showed that LCK* was expressed at the expected molecular weight (Fig. 1e).

To demonstrate that LCK* was inactive but could be rendered functional by illumination, we used HEK-293T cells stably expressing the complete TCR complex (HEK-TCR; Supplementary Fig. 2a) and measured ZAP70 phosphorylation by LCK* upon illumination of the cells. We have previously shown that LCK shows constitutive activity toward the TCR and other downstream signaling molecules in HEK-293T$_{26}$, owing to the near-complete absence of inhibitory proteins that normally restrain its activity (Supplementary Fig. 2b,c). ZAP70 Y319 is a physiologically substrate of LCK and is not associated with any ZAP70 autocatalytic mechanism$_{30,31}$, thereby making it a good readout for LCK kinase activity. We first optimized the length and intensity of the illumination to uncage pc-Lys in LCK* by measuring Y319 phosphorylation by phospho-western analysis (Fig. 1f). Blot quantification showed that before illumination, no phosphorylation was detectable, but 30-s illumination at 12 mW/cm$^2$ uncaged LCK* sufficiently to cause phosphorylation 15 min post illumination without signal saturation (Fig. 1g). This condition was preferred, as it limited any cytotoxic effects of high-intensity light and allowed us to capture the dynamic range of the phosphorylation events over the assay time course. Results from control experiments confirmed that the observed Y319 phosphorylation was specific to the activity of LCK* uncaged by 350–400-nm light illumination. Phosphorylation was not observed when a catalytically inactive variant of LCK (K273R) was equivalently illuminated or when LCK* was illuminated by different wavelengths of light (500–550 nm) (Supplementary Fig. 1b). Using this optimized system, we measured the kinetics of LCK* activity by quenching the phosphorylation reaction at various time points after pc-Lys uncaging. We then observed the expected increase in Y319 phosphorylation over time (Fig. 1h), and a progress curve of the wild-type enzyme activity in live cells was plotted (Fig. 1i). In subsequent experiments, we decreased the density of time points to improve our throughput to analyze several LCK* variants simultaneously, which did not compromise the experimental results (Supplementary Fig. 3a).

Phosphorylation of LCK active-site loop is essential for its kinase activity

LCK, like many members of the tyrosine kinase family, has an activation loop within the kinase domain (Fig. 2a) that must be phosphorylated at a specific tyrosine residue to ensure full enzymatic activity. However, it remains unresolved whether the unphosphorylated form of LCK retains significant activity in vivo, given a recent in vitro study that showed only a two-fold increase in kinase activity when the active-site loop was phosphorylated$_{14}$. We substituted the tyrosine in the activation loop of LCK with phenylalanine (Y394F), which will abolish its phosphorylation. We found that this substitution...
rendered the kinase essentially inactive (Fig. 2b,c), which was not caused by the endogenous expression of the inhibitory kinase CSK by the HEK-TCR cells (Supplementary Fig. 3b). This finding suggested that when not phosphorylated at Y394, LCK activity is almost completely absent when present in its native cellular environment. Conversely, the Y505F mutation in the C-terminal tail of LCK has been known to substantially increase its kinase activity\textsuperscript{32,33} by removing CSK-mediated phosphorylation of Y505 that drives intramolecular inhibition, and this is what we observed (Fig. 2d). To provide a quantitative comparison between the LCK\textsuperscript{*} variants, we derived the maximal reaction rate from the kinetic data, which should be proportional to the catalytic activity of the kinase, assuming equivalent enzyme and substrate concentrations. This data pointed to the Y505F mutant having an ~8-fold increased reaction rate compared to that of wild-type LCK\textsuperscript{*}, whereas the reaction rate for the Y394F mutant was undetectable (Supplementary Fig. 3c).

We used a complementary assay to provide further evidence for the complete inhibition of LCK in the absence of Y394 phosphorylation. Prior to Y319 phosphorylation, ZAP70 is recruited to the phosphorylated ITAMs of the TCR complex at the plasma membrane, an event that also depends on LCK activity. We used live-cell imaging of ZAP70 recruitment to the cell surface as an alternative means to probe LCK

**Figure 3** Intramolecular SH3 interaction restrains LCK kinase activity. (a–c) Plots of ZAP70 Y319 phosphorylation kinetics by R154K LCK\textsuperscript{*} (a), W97A LCK\textsuperscript{*} (b) and P232A LCK\textsuperscript{*} (c), normalized relative to final time point (15 min) of WT LCK\textsuperscript{*} (dashed curve). (d) ZAP70 Y319 phosphorylation kinetics by WT and mutant LCK\textsuperscript{*} kinases are plotted in one graph for direct comparison. Data were fit using the three-parameter logistic function, and are presented as mean ± s.e.m. from independent experiments (n = 12 for WT LCK\textsuperscript{*}; n = 4 for mutant LCK\textsuperscript{*}). Source data for graphs are in Supplementary Data Set 2.

**Figure 4** Active LCK conformation increases substrate availability for autophosphorylation at Y394. (a) Plot showing kinetics of WT LCK\textsuperscript{*} autophosphorylation at Y394 after photouncaging. (b) Representative western blots showing autophosphorylation at Y394 of both WT LCK\textsuperscript{*} and untagged kinase-inactive LCK (K273R) at the indicated time points after LCK\textsuperscript{*} uncaging. ZAP70-mRuby\textsubscript{2} was not included in these experiments. Dotted lines indicate sections from the same blot but presented at different brightness for visual clarity only. (c) Quantification of blots in b to show kinetics of LCK\textsuperscript{*} and LCK (K273R) autophosphorylation at Y394. (d) Maximal autophosphorylation of Y394 achieved by WT LCK\textsuperscript{*} by varying the illumination period of illumination. Separate plots of Y394 autophosphorylation were fit to derive the maximum asymptote values, which were plotted against illumination period. (e) Kinetics of WT LCK\textsuperscript{*} and Y505F LCK\textsuperscript{*} autophosphorylation at Y394. For all plots except d, data are normalized relative to WT LCK\textsuperscript{*} at 1 min and fit by three-parameter logistic function and presented as mean ± s.e.m. from independent experiments (n = 9 in a; 3 in c; 4 in e). For d, error bars represent 95% confidence interval of estimated parameter value from independent experiments (n = 3). Uncropped blot images are shown in Supplementary Data Set 1. Source data for graphs are in Supplementary Data Set 2.
kinase activity toward the TCR. We used an equivalent transfection strategy to that described above but used HEK-TCR cells expressing higher TCR density (HEK-TCR) to increase the membrane signal (Supplementary Fig. 2d). As expected, ZAP70 was initially localized to the cytoplasm but rapidly translocated to the plasma membrane upon the uncaging of wild-type LCK* (Fig. 2c and Supplementary Video 1). Quantitative image analysis measuring the relative fluorescence intensity of ZAP70 at the plasma membrane showed that it was efficiently recruited to the TCR (Fig. 2f). The Y394F mutation of LCK* caused undetectable ZAP70 recruitment (Fig. 2g and Supplementary Video 1). Conversely, the Y505F LCK* variant showed even faster dynamics (Fig. 2h and Supplementary Video 1), saturating ZAP70 binding within 20 s that subsequently decreased, presumably owing to deleterious effects of the overactive kinase on cell integrity, such as membrane blebbing (Supplementary Video 1). Because the kinetics of ZAP70 recruitment to the membrane did not follow a simple profile, we quantified LCK* activity by determining the time required for half-maximal ZAP70 recruitment, with a shorter time corresponding to increased LCK* function (Supplementary Fig. 3d). The results from these imaging experiments are in strong agreement with the conclusions from the phospho-western experiments.

Intramolecular SH3 interaction restraints LCK kinase activity

The SH2 domain of LCK is known to interact not only with phosphorylated tyrosine motifs in other proteins but also with phosphorylated Y505 intramolecularly, which inhibits LCK activity. The generally agreed-upon explanation for why the Y505F mutation of LCK enhances catalytic activity compared to the wild-type kinase is the disruption of this intramolecular inhibition. We wanted to test whether the converse was true; does disrupting binding by the SH2 domain cause equivalent enhancement of LCK function? The R154K substitution in the SH2 domain of LCK is known to disable binding of this domain to phosphorylated polypeptides. We anticipated that this mutation would mimic the Y505F mutant by releasing intramolecular inhibition. However, the R154K mutation significantly decreased ZAP70 Y319 phosphorylation to ~25% compared to that of the wild type (Fig. 3a and Supplementary Fig. 3c), thus implying that the SH2-domain interaction with binding partners, such as the TCR, is more critical to LCK function than its role in intramolecular inhibition.

The SH3 domain of LCK can also participate in intramolecular binding with the conserved proline-rich (PxxP) motif in the linker region, although this interaction is considerably less well characterized compared to the SH2 interaction. We expected that SH3-mediated intramolecular binding would also repress LCK* kinase activity. We first disrupted a key residue (W97A) in the SH3 domain and found that LCK* kinase activity was substantially increased compared to that of wild-type LCK* (Fig. 3b). This finding suggested that, at least within the transfected HEK-TCR cells, the primary function of the SH3 domain is to inhibit LCK kinase activity rather than to bind another target protein. A prediction from this result is that mutation of the proline-rich motif that interacts with the SH3 domain within LCK should have an equivalent effect on the activity of LCK. Through structural modeling of full-length human LCK based on a related kinase, we identified P232 within the conserved PxxP motif of LCK that should interact with W97 of the SH3 domain (Supplementary Fig. 3e). Mutating this residue (P232A) dramatically increased LCK* kinase activity (Fig. 3c). Both W97A and P232A substitutions led to an equivalent increase in LCK* activity that was quantitatively comparable to that of the Y505F mutant (Fig. 3d and Supplementary Fig. 3c), suggesting that the primary function of the intramolecular SH3-mediated interaction is to restrain LCK catalytic activity.

LCK activation by autophosphorylation

It has been shown that Y394 in the activation loop of LCK is autophosphorylated in trans12,14,35,36 but the kinetics of this reaction have not been studied in situ. To address this, we first investigated the kinetics of LCK* Y394 autophosphorylation following pc-Lys uncaging. We found that the process occurred at a much faster rate compared to that of ZAP70 Y319 phosphorylation (Fig. 4a). To confirm that this rapid autophosphorylation was occurring predominantly in trans, we coexpressed wild-type LCK* and a catalytically-inactive variant of LCK (K273R) in cells and measured the phosphorylation of Y394 in both forms by LCK* (Fig. 4b). Because LCK (K273R) is devoid of kinase activity, any phosphorylation observed could only have been attributed to the in trans activity of uncaged LCK*, which was indeed the case (Fig. 4c). However, although the relative rates of Y394 autophosphorylation on both kinase variants were very similar, the absolute level of LCK (K273R) phosphorylation was much lower compared to that of uncaged LCK* (Fig. 4c), which was not owing to differences in their expression (Fig. 4b).

The preceding results suggested that the catalytically-inactive LCK (K273R) was less susceptible to autophosphorylation at Y394, perhaps by adopting a conformation in which Y394 was less accessible, compared to that of the uncaged LCK*. We tested this hypothesis by varying the illumination period used to uncage LCK*. Cells exposed to shorter illumination would have less uncaged, active LCK*. As predicted, shorter illumination led to a lower maximal pY394 signal compared to those of the samples that were illuminated for longer (Fig. 4d). If the effect of different illumination periods was to only vary the level of LCK* as an active enzyme and not as a ‘substrate’, there would have been differences in the rate of phosphorylation and not the maximal phosphorylation, but this is not what we observed. We hypothesized that because the Y505F LCK* mutant should be in a more ‘open’ conformation, it should have increased accessibility of Y394, which is exactly what we observed (Fig. 4e).

ZAP70 depends on autophosphorylation for activation

Full activation of ZAP70 kinase requires phosphorylation of its activation loop at Y493, in an equivalent manner to that of LCK17. Although it is accepted that ZAP70 Y493 phosphorylation requires the presence of active LCK, it is less clear whether LCK itself directly phosphorylates...
H cell expressing LCK* and ZAP70-mRuby2 formed ZAP70 autophosphorylation. We used our LCK* uncaging approach to follow the kinetics of ZAP70 phosphorylation at both Y319 and Y493. We found that LCK*–mediated Y319 phosphorylation had significantly faster kinetics than those of Y493 phosphorylation, with a delay evident between the initiation of ZAP70 Y319 and Y493 phosphorylation (Fig. 5a). We speculated that this difference was due to Y493 phosphorylation being mediated by ZAP70 autophosphorylation rather than LCK*. To investigate this, we repeated the kinetic assay but included a kinase-dead version of ZAP70 (K369R). Compared to wild-type ZAP70, the catalytically inactive version had a far lower rate of Y493 phosphorylation (Fig. 5b). The data show that the primary kinase responsible for ZAP70 Y493 phosphorylation is ZAP70 itself.

Activity of other SRC-family kinases expressed in T cells

There are nine members of the SFK family, but only three are expressed at significant levels in T cells: LCK, FYN and, to a lesser extent, SRC. Although both LCK and FYN play roles during early TCR signaling, it is believed that LCK is the major kinase involved in TCR-mediated signaling. To test whether this was because LCK was more efficient at initiating T-cell signaling, we constructed the photocaged versions of FYN and SRC by mutating the codons for K299 (FYN*) and K298 (SRC*) to the amber stop codon, in an equivalent manner as that for LCK*. We found that ZAP70 Y319 is phosphorylated by LCK* at a much greater rate when compared with FYN* or SRC* (Supplementary Fig. 4), demonstrating that ZAP70 is a better substrate for LCK.

Spatial control of LCK activation using the photocaged system

To demonstrate that we could both spatially and temporally control LCK activation, we took advantage of a cell–cell conjugation system in which the TCR on the HEK cells interacted with its specific peptide (derived from NY-ESO) covalently bound into the MHC protein and expressed on the surface of Raji B cells, which act as efficient ligand-presenting cells. The inhibitory proteins CD45 and CSK were also expressed in HEK-TCR cells to prevent TCR triggering outside the conjugate interface, where cells adhere through coexpression of ICAM-1, which can interact with the integrin LFA-1 on Raji B cells and the TCR–pMHC interaction itself. We uncaged LCK* by illuminating a diffraction-limited (<1 μm) region of the conjugate interface with a 405-nm laser beam, pulsed for 1 ms. This spatiotemporally localized activation of LCK* initially led to the recruitment of ZAP70 to only this region before increasing throughout the cell interface, presumably owing to the lateral diffusion of LCK* (Fig. 6 and Supplementary Video 2). Fortuitously, the presented LCK*-expressing cell was conjugated to two separate B cells, which allowed us to confirm the spatial restriction of LCK* uncaging, because no ZAP70 was recruited to the untargeted conjugate interface (Fig. 6 and Supplementary Video 2). To confirm that this conjugate region nonetheless had the potential to be activated, we subsequently used global illumination and observed ZAP70 recruitment to the second conjugate interface (Fig. 6). This demonstrated that we could both spatially and temporally control LCK activation at subcellular and millisecond resolution in live cells.

Effect of CD4 and CD8 coreceptors on LCK function

It has been established that the T-cell coreceptors have a significant influence on thymocyte development and T-cell activation, and the interaction between the cytosolic tails of the membrane-bound coreceptors and the SH4 domain of LCK (Fig. 2a) has been confirmed. In addition to the intracellular LCK interaction, the coreceptors bind the pMHC protein expressed on antigen-presenting cells. It remains uncertain whether the coreceptor’s effect on LCK activity is mediated solely by influencing the localization, or dwell time, of the kinase at the engaged TCR complex, a question that we sought to directly address using the approaches described above. We first used our cell-conjugate assay to investigate how CD8 modulates LCK*-mediated ZAP70 membrane recruitment. LCK* uncaging caused the translocation of ZAP70 to the entire conjugate site (Fig. 7a and Supplementary Video 3).
Figure 7 T-cell coreceptors CD4 and CD8 directly enhance LCK activity. (a) Representative microscopy images of ZAP70 recruitment to the conjugate interface between HEK-TCR<sup>CD8+</sup> and APCs (Raji B cells) over time after WT LCK* photouncaging. Colored boxes denote protein representation in the overlay image; green is LCK* (not shown). Outline of APC is shown as dotted line. Scale bar, 5 μm. (b) Quantification of ZAP70 recruitment to the conjugate interface over time, as depicted in (a), in HEK-TCR<sup>CD8+</sup> and CD8<sup>-</sup> HEK-TCR<sup>CD8+</sup> cells after LCK* uncaging. (c) Representative microscopy images of accumulation of eGFP-tagged proteins, as illustrated within the panel, at the interface between HEK-TCR<sup>CD8+</sup> and CD8<sup>-</sup>HEK-TCR<sup>CD8+</sup> and APC. The ‘Myr’ control used the first 12 amino acids of the LCK sequence fused to eGFP to target the fluorophore to the plasma membrane by myristoylation and palmitoylation. The ‘CaaX’ control used the C-terminal sequence from K-RAS, which is prenylated to target the fused eGFP to the plasma membrane. Outline of APC is shown as dotted line. Scale bar, 5 μm. (d) Ratio of fluorescence intensity of eGFP-tagged protein inside the conjugate region over that outside the interface in HEK cells. Data are presented as mean ± s.e.m. of 25–45 cells in each condition from three independent experiments. (e–h) Microscopy image quantification showing ZAP70 recruitment to plasma membrane after LCK* uncaging in the absence or presence of CD8<sup>-</sup> (e, g), CD8<sup>+</sup> (f, h), CD8<sup>-</sup>ExCD8<sup>Int</sup> (g), and CD8<sup>-</sup>ExCD8<sup>Int</sup> (h) in HEK-TCR<sup>CD8+</sup> cells. Cells were exposed to light at 5 mW/cm<sup>2</sup> for 2 s (a,b,e–h). Data in b,e–h are normalized to maximum asymptote values for each data set. Lines show data smoothed using a moving-average filter, data points represent mean, and filled areas represent s.e.m. from independent experiments (n = 4 for all conditions), in which 4–8 cells were used in each independent experiment. Source data for graphs are in Supplementary Data Set 2.

Supplementary Video 3), the kinetics of which were quantified over many conjugates (Fig. 7b). Repeating the assay using CD8<sup>+</sup> HEK-TCR<sup>CD8+</sup> cells (Supplementary Fig. 2e) caused an increased rate of ZAP70 translocation following LCK* uncaging (Fig. 7b) and Supplementary Fig. 5a). An equivalent CD4 assay was not possible because the TCR used is restricted by class-I MHC. CD8 could potentially enhance LCK activity by simply increasing the kinase density within the conjugate interface. We did indeed observe increased LCK* in this region compared to the rest of the plasma membrane, but this effect was not CD8 dependent (Fig. 7c,d). Instructively, the lipid-modified N terminus of LCK alone (Myr-eGFP) was sufficient to replicate the increased LCK* density in the conjugate interface (Fig. 7c,d), as was a prenylated eGFP (eGFP-CaaX), which suggested an alteration to the membrane lipid environment around the clustered TCR as the primary cause. To test whether coreceptor binding directly enhanced LCK kinase activity independently from pMHC binding, we repeated the ZAP70 membrane recruitment assay in unconjugated CD8-expressing HEK-TCR<sup>CD8+</sup> cells. CD8 expression still enhanced the activity of LCK* (Fig. 7e and Supplementary Fig. 5b), which we also found for CD4 (Fig. 7f and Supplementary Fig. 5c), suggesting that both coreceptors use a similar mechanism to enhance LCK function.

The possibility remained that the observed results arose from a direct interaction of the coreceptors with the TCR complex itself, as previously suggested for CD4 (ref. 43). To test this hypothesis, we created chimeric coreceptors (CD8<sup>-</sup>ExCD8<sup>Int</sup> and CD8<sup>-</sup>ExCD4<sup>Int</sup>) by substituting their extracellular domains with the monomer CD86 (ref. 44), while maintaining their interaction with LCK. Repeating the ZAP70 recruitment experiment with these constructs showed that the increased LCK<sup>*</sup> activity on coreceptor binding could be entirely attributed to the intracellular LCK<sup>*</sup> interaction (Fig. 7g,h and Supplementary Fig. 5d,e). Furthermore, the coreceptor effect on LCK<sup>*</sup> function was almost completely attenuated when a larger fraction of LCK<sup>*</sup> was uncaged (Supplementary Fig. 5f–i), which agrees with previous work that coreceptor enhancement of TCR signaling is only observed at physiologically low levels of ligand binding<sup>45</sup>.

DISCUSSION

By developing a means to both spatially and temporally activate tyrosine kinases in live cells, we have been able to quantitatively measure the kinetics of LCK catalytic activity in situ, with a level of control over kinase activity not readily achievable by conventional in vivo and in vitro approaches, as previously demonstrated<sup>28</sup>. In agreement with earlier biochemical studies<sup>11–13</sup>, we found that LCK can adopt a range of conformations that have a strong influence on its intrinsic kinase activity<sup>46</sup>. These conformations are primarily driven by intramolecular interactions mediated by both the regulatory SH2 and SH3 domains. We showed that the kinase activity of LCK<sup>*</sup> mutants Y505E, W97A and P232A were quantitatively equivalent (Fig. 3d and Supplementary Fig. 3c), implying that they all biased the kinase structure to the same ‘open’ conformation. However, the concordance of reciprocal mutations affecting the SH3 intramolecular interaction...
Phosphorylation of the active-site loop of LCK leads to rearrangement and stabilization of the catalytic residues into their optimal positions and drives the full activation of the kinase.6,7,49 We found that this phosphorylation was completely dispensable for LCK kinase activity, as the Y394F mutant was essentially inert in both of our in situ assays. This is at odds with a recent in vitro study14 that found only a two-fold difference on Y394 phosphorylation, which could be explained by the additional constraints placed on LCK when localized to the plasma membrane. The kinetics of Y394 autophosphorylation were rapid, saturating approximately 1 min after LCK* photouncaging, which we hypothesize is required to ensure a kinetic advantage for LCK activation over the abundant phosphatases such as CD45 that would antagonize this modification29, as suggested previously12. This result was in striking contrast to the delayed ZAP70 autophosphorylation we measured, which could be rate limiting in TCR triggering and may have a role in ligand discrimination through kinetic proofreading. The decreased Y394 phosphorylation on a kinase-inactive version of LCK (K273R) suggested that LCK in an active, open conformation is a better substrate for autophosphorylation at Y394. This is supported by structural data of various inactive states of SRC kinase domains that show sequestration of Y394 within the active site, thus preventing its autophosphorylation6. A recent study had also suggested that a negative-feedback loop may control LCK activity through Y192 phosphorylation by ZAP70 (ref. 51).

Finally, we found that the T-cell coreceptors have a stimulatory effect on LCK kinase activity, even in the absence of pMHC binding. This effect could be ascribed solely to the intracellular interaction between the coreceptors and LCK (Fig. 7), a result that cannot easily be explained by current models of coreceptor function45,52. We speculate that the physical interaction between LCK and the coreceptors induces a conformational rearrangement that enhances LCK function and implies that the unique SH4 domain of LCK could also form an intramolecular interaction that restrains kinase activity and that is released on coreceptor binding.

Naturally, there are caveats to the approaches in this study. Although assaying kinase function within the cellular context provides a more physiological representation of the environment in situ, this means that the system is less ‘clean’ than true in vitro experiments, because endogenous proteins have the potential to affect our results. We used a nonimmune cell line to mitigate as many of these confounding effects as possible. Another limitation is that the absolute concentrations of the active enzyme and substrate cannot easily be determined, forcing us to measure relative rather than absolute enzyme kinetics. The extremely rapid autophosphorylation of LCK* at Y394 (Fig. 4) and ZAP70 recruitment to the plasma membrane by Y505F LCK* (Fig. 2e–h) upon uncaging indicate that the presence of p-c-Lys within the active site of LCK* did not result in a gross conformational change of the enzyme and that the observed kinetics reflect the true substrate phosphorylation kinetics rather than the recovery of kinase conformation upon uncaging.

In summary, our quantitative investigation of LCK kinase activity has provided new insights into how this important SFK function, with the conclusions likely to translate to other members of the family. Furthermore, the effect of the coreceptors on LCK activity we observed showcases the power of measuring enzyme kinetics in situ, where the modulatory effect of membrane-bound interacting proteins can now be directly addressed. We demonstrated that this approach can be applied to other members of the SFK family (Supplementary Fig. 4) and can easily be extended to many other kinases that contain a critical lysine within their active site, including ZAP70 kinase. Further developments to express the photocaged proteins in other more physiological cell types, such as primary T cells, are ongoing and will enable us to perform in vivo biochemistry experiments to modulate protein function with unprecedented spatiotemporal control.

METHODS
Methods, including statements of data availability and any associated access codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACNOWLEDGMENTS
This work was supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant Number: 099966/Z/12/Z to J.R.J.) and by the Medical Research Council, UK (MC_U105181009 and MC_UP_A024_1008 to J.W.C.). We would like to thank the engineering workshop at the MRC-LMB for manufacturing the illumination device used in this study.

AUTHOR CONTRIBUTIONS
A.L.-J., B.L.M. and J.R.J. designed and performed all of the experiments in the study. A.L.-J. and J.R.J. analyzed the data. M.M. synthesized the unnatural amino acid p-c-Lys. J.W.C. provided scientific input and helped revise the manuscript, which was written by A.L.-J. and J.R.J. All authors contributed to the final manuscript. J.R.J. oversaw and supervised the research.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Smith-GarVIN, J.E., KoretZky, G.A. & Jordan, M.S. T cell activation. Annu. Rev. Immunol. 27, 591–619 (2009).
2. van der Merwe, P.A. & Dushek, O. Mechanisms for T cell receptor triggering. Nat. Rev. Immunol. 11, 47–55 (2011).
3. Ohashi, P.S. T-cell signalling and autoimmunity: molecular mechanisms of disease. Nat. Rev. Immunol. 2, 427–438 (2002).
4. Talab, F., Allen, J.C., Thompson, V., Lin, K. & Slupsy, J.R. LCK is an important mediator of B-cell receptor signaling in chronic lymphocytic leukemia cells. Mol. Cancer Res. 11, 541–554 (2013).
5. Tycko, B., Smith, S.D. & Sklar, J. Chromosomal translocations joining LCK and TCRβ loci in human T cell leukemia. J. Exp. Med. 174, 867–873 (1991).
6. Xu, W., Doshi, A., Lee, M., Eck, M.J. & Harrison, S.C. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol. Cell 3, 629–638 (1999).
7. Boggon, T.J. & Eck, M.J. Structure and regulation of SRC family kinases. Oncogene 23, 7918–7927 (2004).
8. Bogman, M. et al. The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. EMBO J. 11, 2919–2924 (1992).
9. Chow, L.M., Fournel, M., Davidson, D. & Veillette, A. Negative regulation of T-cell receptor signalling by tyrosine kinase protein p50csk. Nature 365, 156–160 (1993).
10. Saunders, A.E. & Johnson, P. Modulation of immune cell signalling by the leucocyte common tyrosine phosphatase, CD45. Cell. Signal. 22, 339–348 (2010).
11. Paster, W. et al. Genetically encoded Förster resonance energy transfer sensors for the conformation of the SRC family kinase Lck. J. Immunol. 182, 2160–2167 (2009).
12. Nika, K. et al. Constitutively active Lck kinase in T cells drives antigen receptor signal transduction. Immunity 32, 766–777 (2010).
13. Stirweiss, A. et al. T cell activation results in conformational changes in the SRC family kinase Lck to induce its activation. Sci. Signal. 6, ra13 (2013).
14. Hui, E. & Vale, R.D. In vitro membrane reconstitution of the T-cell receptor proximal signaling network. Nat. Struct. Mol. Biol. 21, 133–142 (2014).
15. Palacios, E.H. & Weiss, A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene 23, 7990–8000 (2004).
16. Germain, R.N. T-cell development and the CD4-CD8 lineage decision. Nat. Rev. Immunol. 2, 309–322 (2002).
17. Veillette, A., Bookman, M.A., Horak, E.M. & Bolen, J.B. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56{\textsuperscript{ck}}. *Cell* 55, 301–308 (1988).

18. Rudd, C.E., Trevigliani, J.M., Dasgupta, J.D., Wong, L.L. & Schlossman, S.F. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (p58) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA* 85, 5190–5194 (1988).

19. Kim, P.W., Suri, Z.Y., Blacklow, S.C., Wagner, G. & Eck, M.J. A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. *Science* 301, 1725–1728 (2003).

20. Dagliyan, O. et al. Engineering extrinsic disorder to control protein activity in living cells. *Science* 354, 1441–1444 (2016).

21. Karginov, A.V., Ding, F., Kota, P., Dokholyan, N.V. & Hahn, K.M. Engineered allosteric activation of kinases in living cells. *Nat. Biotechnol.* 28, 743–747 (2010).

22. Zhou, X.X., Fan, L.Z., Li, P., Shen, K. & Lin, M.Z. Optical control of cell signaling by single-chain photoswitchable kinases. *Science* 355, 836–842 (2017).

23. Davis, L. & Chin, J.W. Designer proteins: applications of genetic code expansion in cell biology. *Nat. Rev. Mol. Cell Biol.* 13, 168–182 (2012).

24. Chir, J.W. Expanding and reprogramming the genetic code of cells and animals. *Annu. Rev. Biochem.* 83, 379–408 (2014).

25. Gautier, A. et al. Genetically encoded photocontrol of protein localization in mammalian cells. *J. Am. Chem. Soc.* 132, 4086–4088 (2010).

26. James, J.R. & Vale, R.D. Biophysical mechanism of T-cell receptor triggering in a reconstituted system. *Nature* 487, 64–69 (2012).

27. Carrera, A.C., Alexandrov, K. & Roberts, T.M. The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. *Proc. Natl. Acad. Sci. USA* 90, 442–446 (1993).

28. Gautier, A., Deiters, A. & Chin, J.W. Light-activated kinases enable temporal dissection of signaling networks in living cells. *J. Am. Chem. Soc.* 133, 2124–2127 (2011).

29. Schmied, W.H., Eilsasser, S.J., Uttamapinant, C. & Chin, J.W. Efficient multisite unnatural amino acid incorporation in mammalian cells via optimized pyrrolysyl tRNA synthetase/tRNA expression and engineered eRF{\textsubscript{1}}. *J. Am. Chem. Soc.* 136, 15577–15583 (2014).

30. Williams, B.L. et al. Phosphorylation of Tyr{\textsuperscript{319}} in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C{\textsubscript{γ1}} and Ras activation. *EMBO J.* 18, 1832–1844 (1999).

31. Brdicka, T., Kadlec, T.A., Roose, J.P., Pastuszak, A.W. & Weiss, A. Intramolecular regulatory switch in ZAP-70: analogy with receptor tyrosine kinases. *Mol. Cell. Biol.* 25, 4924–4933 (2005).

32. Amrein, K.E. & Setton, B.M. Mutation of a site of tyrosine phosphorylation in the lymphocyte-specific tyrosine protein kinase, p56{\textsuperscript{ck}}, reveals its oncogenic potential in fibroblasts. *Proc. Natl. Acad. Sci. USA* 85, 4247–4251 (1988).

33. Abraham, N. & Veillette, A. Activation of p56{\textsuperscript{ck}} through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autoprophosphorylation and myristylation. *Mol. Cell. Biol.* 10, 5197–5206 (1990).

34. Rossy, J., Owen, D.M., Williamson, D.J., Yang, Z. & Gaus, K. Conformational states of the kinase Lck regulate clustering in early T cell signaling. *Nat. Immunol.* 14, 82–89 (2013).

35. Caron, L., Abraham, N., Pawson, T. & Veillette, A. Structural requirements for enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase p56{\textsuperscript{ck}}. *Mol. Cell. Biol.* 12, 2720–2729 (1992).

36. Luo, K.X. & Setton, B.M. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the Lck tyrosine protein kinase at the autophosphorylation site. *Mol. Cell. Biol.* 10, 5305–5313 (1990).

37. Chan, A.C. et al. Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. *EMBO J.* 14, 2499–2508 (1995).

38. Watts, J.D. et al. Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. *J. Biol. Chem.* 269, 29520–29529 (1994).

39. Kong, G. et al. Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. *Mol. Cell. Biol.* 16, 5026–5035 (1996).

40. Denny, M.F., Patai, B. & Straus, D.B. Differential T-cell antigen receptor signaling mediated by the Src family kinases Lck and Fyn. *Mol. Cell. Biol.* 20, 1426–1435 (2000).

41. Lovatt, M. et al. Lck regulates the threshold of activation in primary T cells, while both Lck and Fyn contribute to the magnitude of the extracellular signal-related kinase response. *Mol. Cell. Biol.* 26, 8655–8665 (2006).

42. Huse, M., Eck, M.J. & Harrison, S.C. A Zn{\textsuperscript{2+}} ion links the cytoplasmic tail of CD4 and the N-terminal region of Lck. *J. Biol. Chem.* 273, 18729–18733 (1998).

43. Vignali, D.A., Carson, R.T., Chang, B., Mittler, R.S. & Strominger, J.L. The two membrane proximal domains of CD4 interact with the T cell receptor. *J. Exp. Med.* 183, 2097–2107 (1996).

44. James, J.R., Oliveira, M.J., Carmo, A.M., Iaboni, A. & Davis, S.J. A rigorous experimental framework for detecting protein oligomerization using bioluminescence resonance energy transfer. *Nat. Methods* 3, 1001–1006 (2006).

45. Artymov, M.N., Lis, M., Devadas, S., Davis, M.M. & Chakraborty, A.K. CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. *Proc. Natl. Acad. Sci. USA* 107, 16916–16921 (2010).

46. Brown, M.T. & Cooper, J.A. Regulation, substrates and functions of src. *Biochim. Biophys. Acta* 1287, 121–149 (1996).

47. Xu, B. & Miller, W.T. Src homology domains of v-Src stabilize an active conformation of the tyrosine kinase catalytic domain. *Mol. Cell. Biochem.* 158, 57–63 (1996).

48. Straus, D.B., Chan, A.C., Patai, B. & Weiss, A. SH2 domain function is essential for the role of the Lck tyrosine kinase in T cell receptor signal transduction. *J. Biol. Chem.* 271, 9976–9981 (1996).

49. Yamaguchi, H. & Hendrickson, W.A. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. *Nature* 384, 484–489 (1996).

50. D’Oro, U., Sakaguchi, K., Appella, E. & Ashwell, J.D. Mutational analysis of Lck in CD45-negative T cells: dominant role of tyrosine 394 phosphorylation in kinase activity. *Mol. Cell. Biol.* 16, 4996–5003 (1996).

51. Coutrey, A.H. et al. Phosphorylation within the SH2 Domain of Lck regulates its activation by CD45. *Mol. Cell* 67, 498–511.e6 (2017).

52. Stepanek, O. et al. Coreceptor scanning by the T cell receptor provides a mechanism for T cell tolerance. *Cell* 159, 333–345 (2014).
Vector constructs. All constructs, unless described otherwise, were created by amplifying gene sequences using PCR, incorporating MluI and BamHI-STOP-NorI restriction sites at the 5′ and 3′ ends of the gene, respectively. Following a double digestion with MluI and NorI, the PCR product was ligated into pHRI-LCK*, (U6-PylT*)4-EF1α-PylRS, pCDNA5-eRF1 (E55D) and pHR-CD45RO vector as described above.

The components required for UAA incorporation of photocaged lysine (pc-Lys) in proteins expressed by mammalian cells have been described previously. Briefly, the orthogonal pair of rNAUUA (U25C) and aminocyclo-tyrosine synthetase required to charge the rNAUUA with photocaged lysine (pc-Lys) are encoded within a PiggyBac vector, (U6-PylT*)4-PylIR. This plasmid contains four copies of the rNAUUA along with the synthetase, under the control of the U6 and EF1α promoter, respectively. The E55D mutant of eukaryotic release factor (eRF1) was additionally used to increase efficiency of pc-Lys incorporation.

For pHRI-LCK*, the IFP2.0 fluorescent protein was fused at the C terminus to the K-Ras prenylation sequence (LEKMSKDGGKKKSKTKCVCYM). The gene was inserted between the BamHI and NorI of the pHRI-LCK vector.

For pHCM-CD45RO, the gene for CD45RO (an N-terminal His tag) was amplified from a published construct. The SFFV promoter was replaced with the CMV equivalent. To drive high expression, the SV40 introns (16S) were synthesized as a GeneArt String and inserted as BstHI-MluI fragment at the 5′ end of CD45RO gene.

For pHRI-CBP-2A-CSK, the genes for CBP and CSK were amplified from pHRI-CBP and pHRI-CSK vectors and inserted in-frame into a bicistronic vector with the P2A self-cleaving peptide sequence linking them.

For pHRI-CD8β(βεζ), the genes for CD8β and CD8ε were amplified from gene-synthesized fragments and inserted in-frame into a bicistronic vector with the P2A self-cleaving peptide sequence linking them.

For pHRI-CDB6(Cαδ) and pHRI-CDB6(Cαδ)¢, the chimeric sequences were gene synthesized (Thermo Fisher Scientific) to fuse the extracellular domain of CD8β with the transmembrane domain and cytosolic tail of CD4 or CD8ε. The synthesized genes were inserted into pHRI vector as described above.

Cell culture. HEK-TCR and HEK-TCRHI cell lines were created by lentiviral transduction of HEK-293T cell line (purchased from ATCC to ensure identity) by stable expression of TCROβδ chains. The MCMV exogenous single-cell suspension was transfected into HEK-293T cells at a ratio of 2:2:1. A total of 1.5 µg of DNA was transfected per well using GeneJuice (Merck). After 48–72 h, the lentivirus-containing medium was collected, centrifuged at 4,300g for 2 min to remove debris, and incubated with HEK cells to be transduced for 16 h. Fresh medium was then added to the cells and expression of the protein of interest was monitored regularly, and the cell population was expanded. Once the expression level was stable, the cells were bulk sorted to isolate cell populations with the desired protein expression level.

Drug treatment. Cells were treated with cytokines as described in the methods. Transfected HEK-293T cells were treated with 100 ng/ml IL-2 or 200 ng/ml IL-15 to enhance IFP2.0 fluorescence. For microscopy experiments of untagged HEK cells, pHRI-LCK*, pCDNA5-eRF1 (E55D) and pHR-ZAP70-mRuby2 at a DNA ratio of 2:2:1. Additional components were included in the mix with a ratio of 1 while maintaining the total of 2 µg DNA per transfection. For microscopy experiments of untagged HEK cells, pHRI-LCK*, pCDNA5-eRF1 (E55D) and pHR-ZAP70-mRuby2 at a DNA ratio of 2:2:1. Additional components were included in the mix with a ratio of 1 while maintaining the total of 2 µg DNA per transfection. For microscopy experiments of untagged HEK cells, pHRI-LCK, (U6-PylT*)4-PylIR, pHRI-LCK, (U6-PylT*)4-PylIR, pCDNA5-eRF1 (E55D) and pHR-ZAP70-mRuby2 at a DNA ratio of 2:2:1. Additional components were included in the mix with a ratio of 1 while maintaining the total of 2 µg DNA per transfection. For microscopy experiments of untagged HEK cells, pHRI-LCK*, pCDNA5-eRF1 (E55D) and pHR-ZAP70-mRuby2 at a DNA ratio of 2:2:1. Additional components were included in the mix with a ratio of 1 while maintaining the total of 2 µg DNA per transfection.

Western blotting. SDS–PAGE was performed using NuPAGE Novex 4–12% Bis-Tris Midi protein gels (26 wells) and XCell SureLock Midi-Cell Electrophoresis System according to the manufacturer's instructions (Thermo Fisher Scientific). Gel transfer to nitrocellulose was performed using the iBlot device (Thermo Fisher Scientific). The primary antibodies used in this study were: anti-pY319-ZAP70 (cat#: 2701), anti-pY493-ZAP70 (cat#: 2704), anti-pY416-SRC (reacts with LCK-pY394; cat#: 2101), anti-LCK (cat#: 2657), anti-SHP-1 (cat#: 3759), anti-SHP-2 (cat#: 3397) and anti-CSK mAb (cat#: 4980), which were all purchased from Cell Signaling Technology and are all raised in rabbit except anti-LCK, which was raised in mouse. Anti-beta actin mAb was raised in mouse (cat#: AB11003, Abcam). For the combined detection of LCK*, FYN* and SRC*, a mouse mAb against eGFp (cat#: A11120; Life Technologies) was used to give equivalent sensitivity. All primary antibodies were used at 1:1,000 dilution. Secondary antibodies were used at 1:20,000 dilution: anti-rabbit or mouse IgG (H+L) DyLight 800 (cat#: 5151 & 5257; Cell Signaling) and Alexa Fluor 680 goat anti-rabbit or mouse IgG (H+L) (cat#: A21109 & A21058; Life Technologies). The transferred blot membrane was first blocked with 2.5% nonfat milk (Sigma) in Tris-buffered saline (MTBS) before incubation with primary antibody in MTBS including 0.1% Tween-20 (MTBS-T) at 4 °C overnight with agitation. The membrane was then washed three times in TBS-T, then incubated for 1 h in the appropriate secondary antibody at room temperature in MTBS-T before being washed three times in TBS. Fluorescent protein bands were visualized using the Odyssey CLX imaging system (LI-COR) that enables two-color detection at 700 nm and 800 nm on the same blot over a 5-decade linear range.
Flow cytometry. An LSR II flow cytometer (Beckton Dickinson) was used to measure expression levels of proteins in transfected or transduced HEK cells. For detection of fluorescently tagged proteins, the cells were washed once in FACS buffer (2% (v/v) PBS, 0.1% (w/v) NaN₃ in PBS, pH 7.4) and fixed in FACS Fix (1.6% (v/v) formaldehyde, 2% (w/v) glucose, 0.1% (w/v) NaN₃ in PBS, pH 7.4) before flow cytometry experiments. For detection of untagged proteins, the primary antibodies used (all from BioLegend) were Alexa Fluor 647–conjugated anti-TCR (cat#: 306714), Alexa Fluor 488–conjugated anti-CD4 (cat#: 317419) or anti-CD8 (cat#: 301024), and an Alexa Fluor 647–conjugated anti-CD45 (cat#: 304056) that recognizes all isoforms. Antibodies were diluted 1:50 in FACS buffer and incubated with the cells for 30 min on ice. Labeled cells were washed with FACS buffer once, cells were centrifuged at 800g for 5 min and the resuspended pellet was fixed in FACS Fix buffer. Routinely, 20,000 live cells gated by scatter were collected for each analysis.

Cell conjugation. HEK-TCR cells for conjugation experiments were grown and transfected in 6-well plates as described above. After 40–48 h, cells were trypsinized to detach them from the wells for 5 min at 37 °C before neutralization by the addition of an equal volume of fresh medium. Cells were pipetted several times to obtain a homogenous single-cell suspension and collected in a 1.5-ml Eppendorf tube. Approximately 1 × 10⁶ Raji B cells expressing the appropriate pMHC were collected in a separate tube. Both tubes were centrifuged at 800g for 3 min to pellet the cells. Resuspended pellets were then washed in fresh complete DMEM and centrifuged again. After centrifugation, both pellets were resuspended in residual medium and combined to a total volume of ~200 µl with medium in a 0.2-ml PCR tube. The cells were incubated in a 37 °C water bath for 30 min to drive cell conjugation. Following incubation, conjugated cells were washed once in Dulbecco’s PBS with GgCl₃ and MgCl₂ (DPBS), resuspended in 1 ml of DMEM with anti-bleaching medium (Evrogen) and transferred to a MatTek imaging dish.

Spinning disc confocal microscopy. For imaging of unconjugated HEK cells to study ZAP70-mRuby2 recruitment to plasma membrane, cells were seeded directly into the imaging dish and transfected as described above. Prior to imaging, cells were washed gently in DPBS once and 1 ml of imaging medium was added to cover the cells in the dish. All live-cell-imaging experiments in this study used a Nikon Ti inverted microscope in a thermally controlled perfect-focus unit of the microscope was used to correct for axial focus drift due to fluctuations in temperature.

Global photo-uncaging. HEK cells expressing both LCK* and ZAP70-mRuby2 were identified under the microscope based on the expression of the tagged fluorescent proteins. In the cell-conjugation experiment, conjugated cells were identified based on the presence of pMHC-BFP-expressing Raji B cells forming a cup-shaped contact with LCK* and ZAP70-mRuby2–expressing HEK cells. To follow ZAP70-mRuby2 recruitment to the plasma membrane or cell-conjugate interface after LCK* activation, cells were illuminated by light from an AMH-200 light source (Andor) through a DAPI filter cube (excitation at 377 ± 25 nm) in the microscope. A script was written in uManager software to capture 5–10 images before photouncaging by illuminating cells through the objective at 5 mW/cm² or 500 mW/cm² for a duration of 2 s. Following this, images were acquired continuously every ~1.4 s for approximately 2 min. Images from all relevant fluorescence channels were captured throughout the experiment. Images were analyzed as described below. Acquisition parameters were set up to ensure the best dynamic range to avoid signal saturation in the fluorescence channels. Owing to variability in the expression of proteins using transient transfection, individual laser powers were adjusted between experiments while maintaining the exposure times.

Focused photouncaging. To activate LCK* spatially with subcellular accuracy in the cell–cell conjugate region, a similar protocol was used as for the global photouncaging. To uncage LCK*, a defined diffraction-limited spot in the image was excited with a 405-nm laser at full power (1.2 mW at objective), using a dwell-time of 100 µs repeated 10 times. Images were subsequently acquired as described above.

Data analysis. Western blot images. High resolution western blot images from the Odyssey imager were acquired as 32-bit grayscale files for quantification with ImageJ software. Equivalent regions of interest (ROIs) were drawn around the bands of interest to measure the integrated density of each ROI. To express the level of phosphorylation relative to LCK*, for correct to loading and expression, the density value of the phosphorylated band was divided by that of the corresponding LCK* band to obtain the relative phosphorylation of each sample. To allow comparison between experiments, these values were then normalized according to the figure legends to create a plot of phosphorylation kinetics. For every experiment, a WT data set was also collected so that different conditions could be directly compared through this standard. Further processing of the western blots shown in figures (rotation, cropping and contrasting) was performed uniformly over images solely for better visualization. Where needed, white dotted lines denote sections of the same blot with different contrast settings for visual clarity only. Image quantification, however, always used the values from original image files.

Microscopy images. Processing and quantitative analyses of microscopy images were performed using scripts written in ImageJ. Data from the ZAP70-mRuby2 channel were corrected for photobleaching before quantification, which followed a single-exponential decay process. ROIs were drawn around the cell edge or the cell-conjugate region to measure the signal for ZAP70-mRuby2 recruitment to these regions over time. To define the plasma membrane ROIs, images from the ZAP70-mRuby2, LCK*, and IFP2.0-CaaX stacks were combined, background subtracted and contrast enhanced to clearly define a cell shape that could be thresholded to create a binary mask of the cell. This mask was then outlined to define the cell edge as a single-pixel-width ROI, which was remapped to the range [0, 1]. The pixel count in the ROI was used as the mask area. To draw an ROI in the cell-conjugate region, the images from the pMHC-BFP channel were used. Prior to quantification, where needed, image stacks were cropped to isolate the relevant conjugated cells to remove signal from nearby unconjugated cells. Upon background subtraction, contrast enhancement and Gaussian smoothing, the conjugate region was thresholded to create a binary mask. This mask was skeletonized to create a single-pixel-width ROI and mapped over [0, 1]. The masked images and corresponding ZAP70-mRuby2 stack were then multiplied to create a stack that only contained the fluorescence intensity values in the ROI only. To obtain the mean value of mRuby2 intensity in the ROI, the summed intensities were divided by the ROI area. A similar workflow was performed to obtain the mean value of mRuby2 over the entire cell by drawing an ROI of the area bounded by the cell mask. The mean intensity at the cell edge or conjugate region was divided by the corresponding mean intensity over the whole cell to obtain the relative fraction for ZAP70 membrane or conjugate recruitment in each image, plotted against time in the main text. To measure the ratio of eGFP signal inside over outside the conjugate, line ROIs were drawn manually to define the conjugate region and the edge of eGFP-expressing HEK cells before illumination. Mean intensities of eGFP were then measured at these ROIs.

Quantification of endogenous protein expression. Expression of endogenous CSK, SHP-1 and SHP-2 in various cell lines was detected by western blot using monoclonal antibodies specific to the proteins. Samples were prepared as described for the main experiment, and the blots were analyzed as described above. Three different dilutions of each soluble cell lysate were measured to obtain an average value. Raw intensity values from the blots were corrected for the dilution factors obtained from the relative expression of an actin control, cell density in sample and the cytoplasmic volumes of the specific cell lines. These corrected values for each cytoplasmic protein are then presented as relative values compared to the protein expression in CD4⁺ T cells. Cytoplasmic volumes of the different cell lines were 96.1 fl for CD4⁺ and CD8⁺ T cells, 664 fl for Jurkat T cells and 790 fl for HEK293T cells.

Data plotting. All data were initially processed in Excel (Microsoft) to compute the mean and s.e.m. values. Subsequent data plotting was performed using...
MATLAB (MathWorks). Where appropriate, data fitting was performed using a three-parameter logistic function:

\[
y(t) = \frac{d}{1 + \left(\frac{c}{d}\right)^b}
\]

where \(b\) is the Hill's coefficient, \(c\) is the inflection point, \(d\) is the maximum asymptote and \(t\) is time (min). The maximum asymptote values calculated were used for normalization of the imaging data in Figure 7, and the derived 95% confidence interval values were used as the error bar values in Figure 4d. Otherwise, a moving-average filter was used to smooth the data points. Error bars were used to represent the s.e.m. of discrete data points in phosphorylation analyses. Filled area was used to represent the s.e.m. of the more continuous data points in the ZAP70 recruitment analyses.

**Derivation of relative initial rates and time to half-maximal ZAP70 recruitment.**

Plots from western and imaging data were analyzed by MATLAB (MathWorks) further to derive a measure of the rate of LCK kinase activity in each experiment. For the Western analyses, the reaction rate was estimated by differentiating the three-parameter logistic function used to fit the data (see data plotting above) with respect to time. Taking this value at \(t = 0.5\) min defined the initial reaction rate \(\left(V_0\right)\), when this parameter is expected to be maximal immediately after end of the photouncaging period. For the imaging experiments, the ZAP70-recruitment data sets were smoothed and normalized to the corresponding maximum values for data sets that had reached saturation or maximum asymptotes for data sets that had not. The normalized data were then spline fitted to estimate the time when the recruitment of ZAP70 to the plasma membrane was at half-maximum. In both cases, rate parameters were determined from \(n\) number of independent experiments as indicated in the figure legends, and the mean and s.e.m. values were calculated from these values.

**Data availability.** Source data used to generate all graphs presented in this study are available in Supplementary Data Set 2. Other data that support the findings of this study (for example, microscopy images and prenormalization raw values) are available from the corresponding author upon reasonable request. A Life Sciences Reporting Summary for this article is available online.

53. James, J.R. et al. The T cell receptor triggering apparatus is composed of monovalent or monomeric proteins. J. Biol. Chem. 286, 31993–32001 (2011).
54. Tan, C.W. et al. Wnt signalling pathway parameters for mammalian cells. PLoS One 7, e31882 (2012).
55. Rosenbluth, M.J., Lam, W.A. & Fletcher, D.A. Force microscopy of nonadherent cells: a comparison of leukemia cell deformability. Biophys. J. 90, 2994–3003 (2006).
56. Loiko, V.A. et al. Morphometric model of lymphocyte as applied to scanning flow cytometry. J. Quant. Spectrosc. Radiat. Transf. 102, 73–84 (2006).
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work we publish. This form is published with all life science papers and is intended to promote consistency and transparency in reporting. All life sciences submissions use this form; while some list items might not apply to an individual manuscript, all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size
   Describe how sample size was determined.
   All experiments were repeated a minimum of 3 times as biological replicates to ensure reproducibility of the presented results.

2. Data exclusions
   Describe any data exclusions.
   No data was excluded from the analyses.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All experiments were reliably reproduced, including many that were performed by two different researchers separated by > 6 months.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Not required as there was no randomised allocation of samples into experimental groups used.

Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   No blinding, as there was no scoring or any other metric that might be swayed by investigator bias.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

- □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- □ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- □ A statement indicating how many times each experiment was replicated

- □ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- □ The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted
- □ A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- □ □ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

FIJI (ImageJ) was used for all image analysis. MATLAB and Excel were used for all data analysis.

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restrictions.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Source and catalogue numbers are provided for all commercial antibodies used in the methods section. All antibodies were used in assays that were validated by the company, and we used negative controls to confirm the absence of signal when no antigen was present. All antibodies were used to target equivalent species to that the antibodies were raised against.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

All cell lines were originally purchased from ATCC, and is noted in the methods section.

Cells were direct from ATCC, so previously authenticated. For T cells, B cells, defining markers (CD3, CD19) were used routinely to confirm cell type.

All cell lines tested negative for mycoplasma contamination.

HEK-293T are a very standard cell line, purchased directly from ATCC. A justification is provided in the methods section.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human research participants used.