

MOLECULAR BIOLOGY

Biophysical basis underlying dynamic Lck activation visualized by ZapLck FRET biosensor

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Lck plays crucial roles in TCR signaling. We developed a new and sensitive FRET biosensor (ZapLck) to visualize Lck kinase activity with high spatiotemporal resolutions in live cells. ZapLck revealed that 62% of Lck signal was preactivated in T-cells. In Lck-deficient JCam T-cells, Lck preactivation was abolished, which can be restored to 51% by reconstitution with wild-type Lck (LckWT) but not a putatively inactive mutant LckY394F. LckWT also showed a stronger basal Lck-Lck interaction and a slower diffusion rate than LckY394F. Interestingly, aggregation of TCR receptors by antibodies in JCam cells led to a strong activation of reconstituted LckY394F similar to LckWT. Both activated LckY394F and LckWT diffused more slowly and displayed increased Lck-Lck interaction at a similar level. Therefore, these results suggest that a phosphorylatable Y394 is necessary for the basal-level interaction and preactivation of LckWT, while antibody-induced TCR aggregation can trigger the full activation of LckY394F.

INTRODUCTION

T cell receptor (TCR) signals are initiated when foreign antigens are presented to induce the TCR complex formation (1, 2). The first detectable event of TCR signals is that an activated Src family kinase (SFK), lymphocyte-specific protein tyrosine kinase (Lck), phosphorylates the tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) located at the cytoplasmic tails of TCR ζ chain subunits and co-receptor CD3 (1, 2). Phosphorylated ITAMs function as docking sites to recruit the cytoplasmic kinase Zap70 (ζ chain-associated protein 70 kDa) to the plasma membrane for phosphorylation and activation by Lck (3). Active Zap70 then phosphorylates other kinases to transduce downstream signaling cascades, such as the activation of extracellular signal-regulated kinase (ERK) to up-regulate interleukin-2 (IL-2) expressions (4–6). In addition to CD3 stimulation, CD28 costimulation can promote the enrichment of membrane molecular clusters and facilitate T cell proliferation and differentiation (7, 8).

As an early kinase activated upon TCR triggering, Lck plays crucial roles in T cell activation and development (9, 10). Abnormal Lck expression, activation, and transport have been reported to cause severe immune deficiency in humans (11, 12). Overexpression of Lck has been identified in cancers such as lymphoma and leukemia (13). However, the pathophysiological regulations of Lck function at the initiation of TCR signaling remain largely unclear.

As an SFK, Lck has a similar protein structure as other SFK members, containing an N-terminal-specific domain, Src homology 3 (SH3), SH2, a kinase domain, and a C-terminal tail (14, 15). These domains regulate Lck functions via its molecular conformation, kinase-domain accessibility, TCR interaction, and plasma membrane localization (16, 17). Particularly, residues lysine 287 (K287) and tyrosine 394 (Y394) within the Lck kinase domain as well as tyrosine 505 (Y505) at the cy-

toplasmic tail are crucial regulators of Lck kinase activity (18, 19). While modifications of K287 and Y505 have been shown to have activating and inhibitory effects on Lck activity, respectively, the role of Y394 remains unclear. Phosphorylation of Y394 has been suggested to stabilize the open active conformation of Lck and positively contribute to Lck kinase activation, and the tyrosine-to-phenylalanine mutation Y394F has been shown to impair Lck activity (18, 20, 21). However, it has remained controversial how the mutation on Y394 affects Lck kinase activity and whether LckY394F (LckYF) is enzymatically incapable of being activated (21–24). It is also unclear how Y394 phosphorylation affects the biophysical properties that mediate Lck activation and TCR downstream signals (25, 26).

Fluorescence (or Förster) resonance energy transfer (FRET)-based biosensors have been widely used to visualize molecular activities in live cells with high spatiotemporal resolution (27, 28). However, there are currently no broadly applicable Lck biosensors to allow the convenient visualization of the dynamic kinase activity of Lck in live T cells. A fluorescence lifetime imaging microscopy (FLIM)-based, full-length Lck, FRET biosensor has been developed and applied to investigate the regulatory effect of conformational change in Lck, although the biosensor is not able to directly report Lck kinase activity (26, 29). In the current study, we developed a new substrate-based single-chain Lck FRET biosensor (ZapLck) to monitor the spatiotemporal Lck kinase activity in live T cells. This Lck biosensor contains an enhanced cyan fluorescence protein (ECFP) and a variant of yellow fluorescence protein (YPet) as the reporting unit and an SH2 domain and Lck-sensitive tyrosine peptide as the sensing unit (30–32). Being substrate based without an enzymatic domain, our biosensor should not substantially perturb endogenous Lck signals in T cells. With ZapLck, we found that the putatively inactive mutant LckY394F diffused faster than wild-type Lck (LckWT) and exhibited low kinase activity at the basal level. However, both LckY394F and LckWT can be fully activated upon the ligation and clustering of TCRs in live T cells. Further experiments revealed that LckY394F can be activated to the same degree as LckWT and can function to transduce the TCR signal downstream to ERK activation with similar strength and kinetics as LckWT. As such, ZapLck provided a powerful tool to unravel the molecular basis of Lck activation in live cells.

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RESULTS

Engineering and in vitro characterization of a new Lck-FRET-Zap70FY kinase biosensor

On the basis of the principle of FRET, we used a previously designed kinase biosensor cassette for the construction of the Lck FRET biosensors (31, 32). This cassette consists of an ECFP as the donor, an Src SH2 domain with a C185A mutation, a flexible linker, an interchangeable tyrosine substrate of Lck kinase, and a YPet as the acceptor, from the N to the C terminus (Fig. 1A) (31). In this cassette, a biosensor in the resting state is expected to show a high FRET efficiency between the donor and acceptor due to their sticky interface and, hence, a low ECFP/FRET emission ratio (Fig. 1A). We used a mutant Src SH2 domain that has been optimized to bind phosphor-tyrosine-containing peptides with high affinity and reversibility (33). Upon Lck kinase activation, the substrate is phosphorylated and binds to the SH2 domain, causing ECFP and YPet to separate and resulting in a high ECFP/FRET emission ratio (Fig. 1A). Therefore, we will use the ECFP/FRET ratio reported by the biosensor to represent the Lck kinase activity.

To design a highly sensitive and specific Lck biosensor, we identified the optimal tyrosine substrate among known substrates of Lck in sensing its kinase activity. Upon TCR triggering in T cells, active Lck

can phosphorylate the D/ExxYxxI/Lx(6–12)YxxI/L peptide sequences on TCR ζ chain ITAMs (34). The double-phosphorylated ITAM tyrosine peptide then acts as the docking site to recruit an Syk family kinase, Zap70, to the plasma membrane, where Lck then phosphorylates Zap70 residues tyrosine 315 (Y315) and tyrosine 319 (Y319), inducing Zap70 activation and further phosphorylation (35, 36). The Zap70 residue Y319 has also been reported to be essential for TCR signaling, while Y315 is nonessential (37). Hence, we engineered Lck biosensors with substrate peptides containing the Y315 and Y319 tyrosine sites (Zap70YY), or a single mutation of Y315F to promote the primary SH2 binding site pY319 and simplify the design (Zap70FY; Fig. 1B). Among three ITAMs located on the TCR ζ chains, the C-terminal motif is sensitive to Lck phosphorylation and most likely recruits Zap70 upon phosphorylation (38). Since there are two tyrosine residues on the C-terminal motif, two separate substrates encompassing either the first or second tyrosine site were designed (ITAM1 and ITAM2) (Fig. 1B). As Lck can autophosphorylate the Y394 site, we designed another substrate peptide containing Y394 from Lck (Fig. 1B) (14, 24).

The specificity and sensitivity of Lck biosensors with different substrate peptides toward the active SFKs Lck, Fyn, and Src were evaluated by in vitro kinase assay. Our results show that Lck-FRET-Zap70FY and

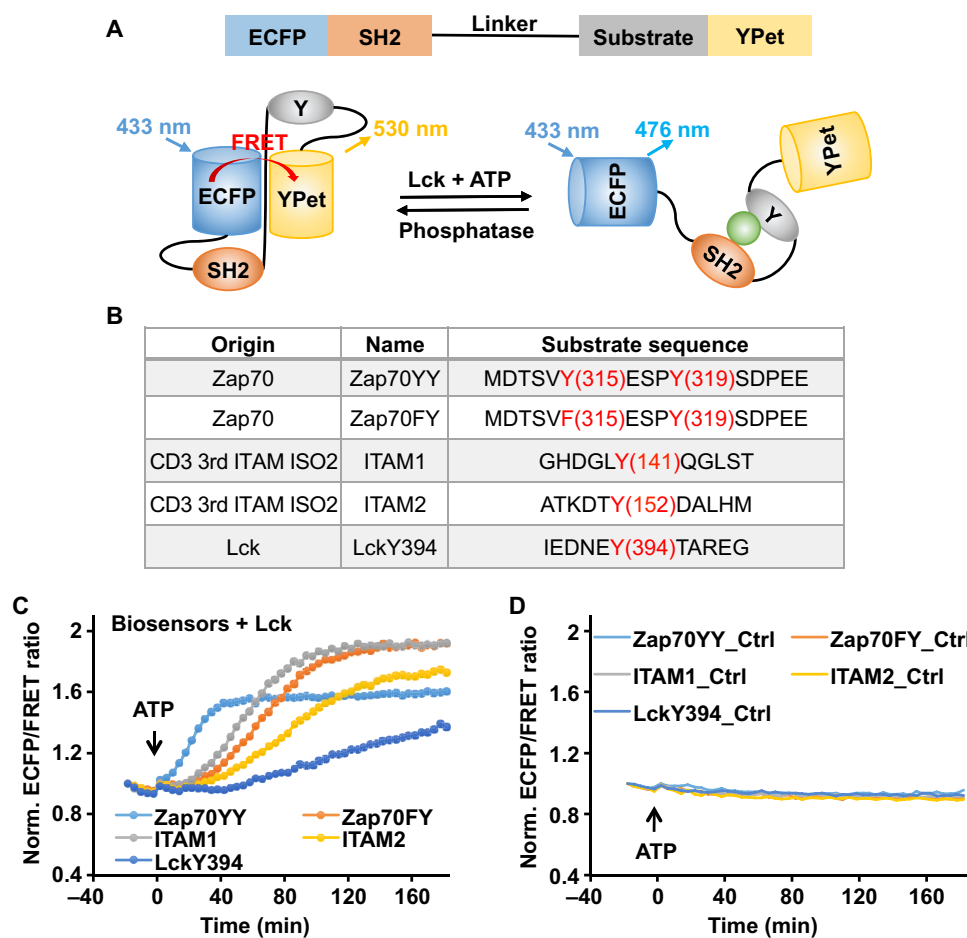


Fig. 1. Lck FRET biosensor design and in vitro characterization. (A) Top: Schematic design of the Lck FRET biosensor. Bottom: Activation mechanism of the substrate-based Lck FRET biosensor. (B) Different substrate peptides tested for Lck FRET biosensors. (C and D) In vitro characterization of Lck FRET biosensors with different substrate peptides premixed with (C) or without (D, Ctrl) active Lck kinase. The panels show normalized time courses of the ECFP/FRET ratio before and after the addition of adenosine 5'-triphosphate (ATP).

Lck-FRET-ITAM1 exhibited the largest increase in ECFP/FRET emission ratio toward Lck kinase activity, while Lck-FRET-Zap70YY was observed to respond fastest among all the biosensors (Fig. 1C). All the biosensors responded to both active Src and Fyn with similar magnitude and kinetics as Lck (fig. S1). These results indicate that the Lck biosensors may be sensitive to the SFK family kinases Src and Fyn in vitro (Fig. 1, C and D). Therefore, the Lck substrate peptides Zap70YY, Zap70FY, and ITAM1 were further examined to identify a sensitive and specific Lck FRET biosensor.

Zap70FY is an ideal substrate for Lck FRET biosensor in cells

To further characterize in cells the sensitivity and specificity of Lck biosensors with the different substrates (Zap70YY, Zap70FY, or ITAM1), HeLa cells coexpressing a biosensor and the Lck or Fyn kinase were activated by an epidermal growth factor (EGF). Upon EGF activation, the ECFP/FRET ratio of the Lck-FRET-Zap70FY biosensor increased 43 and 20% in HeLa cells coexpressing Lck and Fyn, respectively. These responses were inhibited when cells were pretreated with an SFK selective kinase inhibitor, PP1 (fig. S2, A and B). These results indicate that the Lck-FRET-Zap70FY biosensor is specific to SFKs and has higher sensitivity toward the Lck kinase than the Fyn kinase in cells. In comparison, the Lck-FRET-Zap70YY biosensor was less specific in differentiating Lck from Fyn, with a clear response despite inhibition by PP1 (fig. S2C). The Lck-FRET-ITAM1 biosensor showed

a very small ratio increase (fig. S2D). Together, we conclude that the Lck-FRET-Zap70FY biosensor is a good candidate biosensor to monitor Lck kinase activation in live cells with reasonable sensitivity and specificity.

Lck plays crucial roles in TCR signaling transduction in T cells (39, 40), so we further characterized our Lck-FRET-Zap70FY biosensor in T cells by comparing it to a negative control biosensor with dual mutations Y315F and Y319F (Lck-FRET-Zap70FF). We stimulated JurkatE6-1 (Jurkat) cells with conjugated clusters of CD3/CD28 antibodies to initiate TCR signaling and activate Lck (7, 8). Upon stimulation, the ECFP/FRET ratio of the Lck-FRET-Zap70FY biosensor increased to the peak value within 6 min and then leveled off. This activation can be inhibited by PP1, indicating that the ratio change was specific to SFK kinase activity (Fig. 2, A and B, fig. S3, and movie S1). In contrast, the ECFP/FRET ratio of the mutant biosensor, Lck-FRET-Zap70FF, did not change after stimulation, confirming that the biosensor ratio change is mediated by tyrosine phosphorylation as designed (Figs. 1A and 2, A and B) (37). As quantified in Fig. 2C, antibody stimulation induced a significant ~15% ratio increase of our Lck-FRET-Zap70FY FRET biosensor in Jurkat cells, which can be inhibited by PP1 to ~28% less than the basal level. As such, our Lck-FRET-Zap70FY biosensor is sensitive and specific for monitoring Lck kinase activity in live T cells. Hereafter, we refer to the Lck-FRET-Zap70FY biosensor as ZapLck.

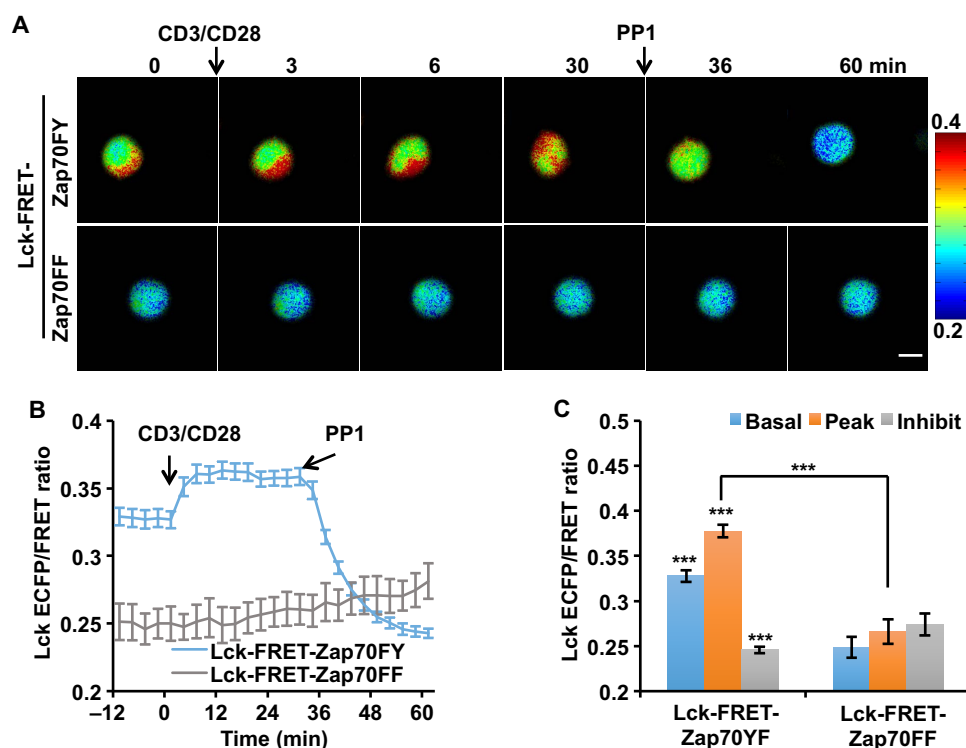


Fig. 2. Characterization of the ZapLck biosensor in live T cells. (A) ECFP/FRET ratio image of representative Jurkat cells transfected with the ZapLck biosensor or its mutant Lck-FRET-Zap70FF. Cells were stimulated with CD3/CD28 antibody clusters (at 10 and 5 $\mu\text{g}/\text{ml}$, respectively, as detailed in Materials and Methods) and then treated with the SFK inhibitor PP1. Scale bar, 5 μm . (B) Quantified time courses comparing the ECFP/FRET ratio change of the Lck biosensor with its mutant in Jurkat cells as shown in (A). (C) Comparison of the basal ratio before stimulation, peak ratio in 30 min after stimulation, and average ratio during 20 to 30 min after PP1 inhibition. The quantified ratio values in cells with the ZapLck biosensor are 0.328 ± 0.007 (basal), 0.378 ± 0.007 (peak), and 0.246 ± 0.004 (inhibit) ($N = 4$, $n = 45$), and those in cells with Lck-FRET-Zap70FF are 0.275 ± 0.015 (basal), 0.286 ± 0.015 (peak), and 0.280 ± 0.015 (inhibit) ($N = 3$, $n = 6$). Error bars: mean $\pm$ SEM. Two-tailed Student's t test was used for statistical analysis. ***Significant difference from other groups in the same cluster or from the indicated group in the other cluster, $P < 1 \times 10^{-3}$. A representative Jurkat cell under treatment is also shown in movie S1.

Assuming that Lck was fully activated by stimulation until it was inhibited by PP1, we use the following formula to estimate the portion of preactivated Lck kinase (Fig. 2C)

$$\frac{\text{Ratio Basal} - \text{Ratio Inhibitor}}{\text{Ratio Peak} - \text{Ratio Inhibitor}} \times 100\%$$

It was therefore estimated that 62% of Lck was preactivated in Jurkat cells at the basal level before stimulation, although it is possible that PP1 inhibition was not sufficient to reduce Lck activity to the lowest level after Jurkat activation. We performed PP1 inhibition and a washout experiment to confirm the basal Lck FRET ratio in Jurkat cells (fig. S4). Jurkat cells directly treated with PP1 showed a ratio value of 0.249 ± 0.007 (fig. S4), similar to the that of the CD3/CD28-stimulated Jurkat cells treated by PP1 (0.246 ± 0.004 ; Fig. 2C), which was used to estimate the preactivated fraction of Lck. This control experiment confirmed that the activation/inhibition assay can be used to estimate the preactivated portion of the Lck kinase with reasonable accuracy, except for the potential limitation that the relationship between FRET ratio signal and amount of active Lck molecule may not be direct. Further experiments show that PP1 inhibition can also significantly reduce the Lck FRET signal in primary peripheral blood mononuclear cells (PBMCs) (fig. S4), indicating that the preactivated portion of the Lck kinase also exists in primary T cells. Furthermore, the different preactivated portions between the cells expressing LckWT-mCherry and LckY394F-mCherry are probably not due to their different subcellular distributions, since the mCherry-tagged LckWT showed similar subcellular localization as mCherry-LckYF (fig. S5), as well as the endogenous Lck revealed by immunostaining (41).

Both the LckWT and LckY394F kinases can be activated to a similar level in live T cells by TCR co-receptor stimulation

To investigate the regulation of Lck kinase function in T cells, we used the new ZapLck biosensor to image Lck-deficient J.Cam1.6 (JCam) T cells derived from Jurkat. When the JCam cells were stimulated with CD3/CD28 antibody clusters, no ECFP/FRET emission ratio change of the biosensor was detected, confirming that this biosensor can be applied to specifically monitor Lck kinase activity in T cells (Fig. 3, A to C, and movie S1).

We then infected the JCam cells with lentiviral LckWT, the kinase-dead Lck with K273R mutation (LckKR), or LckY394F and named the resulting cells JCam-LckWT, JCam-LckKR, and JCam-LckYF, respectively (20, 21, 42). When these cells coexpressing ZapLck biosensors were stimulated with CD3/CD28 antibody clusters, the JCam-LckWT cells showed an average 19% increase in ECFP/FRET emission ratio from the basal level, while JCam-LckYF cells showed an unexpected 40% increase, which reached to a peak value similar to the biosensors in JCam-LckWT cells (Fig. 3). As expected, JCam-LckKR showed no ratio change (Fig. 3, A and B). It is noteworthy that JCam cells expressed LckWT at a low level with an mCherry intensity ranging between 500 and 1500, while LckYF was expressed at a high level with an intensity range of 500 to 8000. To rule out the possibility that the expression levels of the Lck variants may affect the results in Fig. 3, we further quantified time courses of the Lck ECFP/FRET ratio in JCam cells expressing different mCherry-tagged Lck mutants at comparable levels (500 to 1500 in intensity; fig. S3). At this expression level, JCam cells with Lck mutants show similarly different dynamic responses as before (Fig. 3 and fig. S3E). Therefore, these results confirmed that

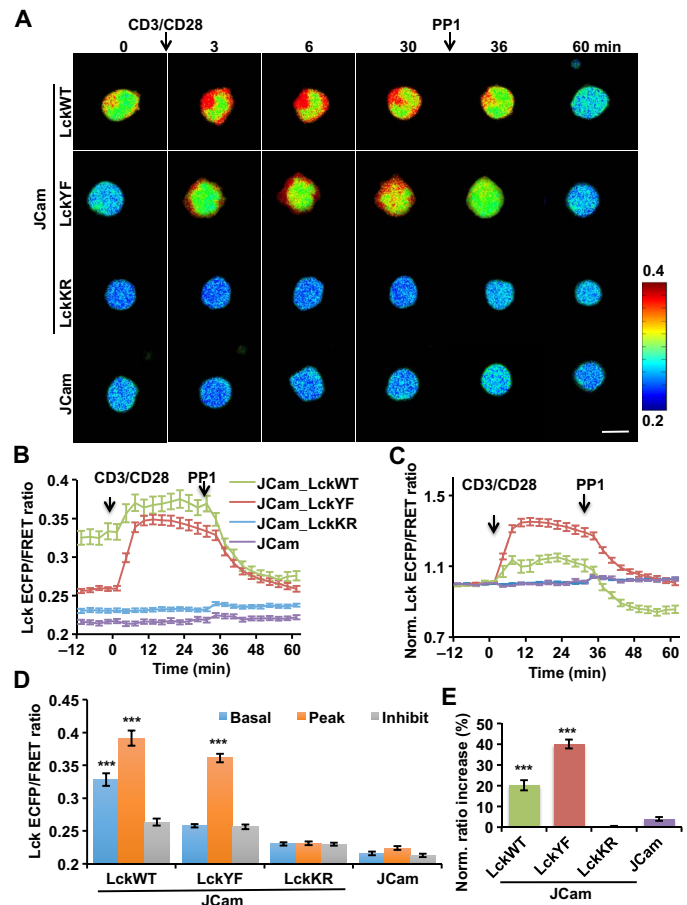


Fig. 3. Lck kinase activities regulated by Y394 in T cells stimulated by CD3/CD28 antibody clusters. The ZapLck biosensor was electroporated into Jurkat or JCam cells reconstituted with LckWT, LckY394F, or LckKR by lentiviral infection. During imaging, the cells were stimulated with CD3/CD28 antibody clusters at 0 min and then inhibited with PP1 at 30 min. (A) ECFP/FRET ratio images of this Lck biosensor in representative JCam cells with different Lck mutants under CD3/CD28 stimulation and PP1 inhibition. Scale bar, 5 μ m. (B) Quantified ECFP/FRET ratio time courses (mean $\pm$ SEM) of the Lck biosensor in live T cells treated as described in (A). (C) Normalized ECFP/FRET ratio time courses shown in (B). (D) Comparison of the basal ECFP/FRET ratio values during 12 min before addition of antibodies (basal), the peak ECFP/FRET ratio values of the Lck biosensors in 30 min after addition of antibodies (peak), and the minimum ECFP/FRET ratio within 30 min after PP1 inhibition (inhibit) in cells shown in (B). $N = 3$, $n = 44$, 77, 60, and 32 for JCam with LckWT, LckY394F, LckKR, and JCam only, respectively. The quantified ECFP/FRET ratio values are 0.328 ± 0.010 (basal), 0.391 ± 0.012 (peak), and 0.263 ± 0.005 (inhibit) for the LckWT group, and 0.258 ± 0.003 (basal), 0.361 ± 0.007 (peak), and 0.256 ± 0.04 (inhibit) for the LckY394F group. Error bars: mean $\pm$ SEM. (E) Comparison of the normalized FRET ratio increase in cells shown in (C). The quantified percentage increase of ratio values are $19.2 \pm 3.7\%$ (LckWT), $39.9 \pm 1.6\%$ (LckY394F), $0.4 \pm 0.3\%$ (LckKR), and $3.7 \pm 1.4\%$ (JCam). The Bonferroni multiple comparison test provided by the multcompare function in the MATLAB statistics toolbox was used for statistical tests. ****Significant difference from all other groups with $P < 1 \times 10^{-3}$. Representative JCam cells with or without different Lck mutants under treatment are also shown in movie S1.

the observed differential biosensor responses in Fig. 3 were mainly caused by different types of Lck variants but not by their expression levels (fig. S3E).

Together, our results indicate that, similar to LckWT, LckY394F has a kinase activity that can be significantly activated by CD3/CD28

co-receptor stimulation in JCam cells. PP1 treatment significantly inhibited the observed increase of the ECFP/FRET ratio in JCam-LckWT and JCam-LckYF cells (Fig. 3, A and B, and fig. S3), indicating that these signals are caused by Lck kinase activation. Although both c-Src and Fyn can activate the Lck biosensor in vitro (fig. S1), the expression of c-Src and Fyn is considered low in T cells (39). Less c-Src and Fyn than Lck were expressed in Jurkat T cells (43). Our Lck biosensor showed activation in stimulated JCam cells reconstituted with LckWT and LckYF, but not in the Lck-deficient JCam cell, although these JCam cells showed similar levels of c-Src/Fyn expression as that of Jurkat cells (Fig. 3) (43). These results suggest that the FRET response of our Lck biosensor is specific to Lck kinase in T cells. Our ZapLck biosensor, hence, provided a new tool in revealing the preactivated portions of Lck. A previously published Src biosensor showed an overall very low ECFP/FRET ratio and did not detect any preactivated kinase in Jurkat cells, although this Src sensor is functional in detecting Src kinase activations since it responded strongly to a pervanadate stimulation in HeLa cells in the control experiment (fig. S5) (31).

Similar to Jurkat cells, before stimulation, the basal Lck activity of JCam-LckWT was significantly higher than that of JCam-LckYF, JCam-LckKR, and JCam cells (Fig. 3, A, B, and D, and movie S1). When treated with PP1, the emission ratio of the biosensor in JCam-LckWT cells, but not that in JCam-LckYF, dropped to a lower level than the basal ratio. Again, assuming that Lck was fully activated by the antibodies and PP1 inhibited all Lck activity, we estimate that 51% of active LckWT were preactivated at the basal level in JCam cells, which is consistent with our observation in Jurkat cells and a previous report (44). In contrast, only ~2% of LckY394F were preactivated, which probably explains why some of the previous publications found LckY394F inactive in T cells, although LckY394F had been reported with kinase activity in *Escherichia coli* (Fig. 3, B and E) (22, 23). The Western blot results further confirmed that LckWT can be quite active with or without co-receptor stimulation, while LckY394F was activated only after stimulation (fig. S3).

To examine whether the Zap70 residue Y319 on the biosensor was phosphorylated by the endogenous Zap70 (37), we performed control experiments in the Zap70-deficient P116 cells (fig. S3F) (45). The Zap70-deficient P116 cells showed similar Lck activation and biosensor responses to CD3/CD28 stimulation as the Jurkat and JCam-LckWT cells, which can be significantly reduced by PP1 inhibition (Figs. 2 and 3 and fig. S3F). These results confirmed that the biosensor responses are unlikely due to the unspecific activation by Zap70.

Together, our ZapLck biosensor showed that LckWT, but not LckY394F, is preactivated in T cells. Although LckY394F has a lower basal activity than LckWT, it can be fully activated upon the ligation and clustering of TCR and co-receptors, suggesting that the Y394 site is crucial for the high Lck kinase activity only under the basal condition, but dispensable in activated T cells.

LckWT and LckY394F transduce fast ERK activation downstream

Since the mutant LckY394F can still respond to TCR co-receptor stimulation, we further examined its ability to transduce signals downstream. We used a FRET-based ERK (NES) biosensor residing outside the nucleus (46) to monitor the dynamic ERK activation in T cells with different Lck mutants, with the FRET/ECFP emission intensity ratio of the ERK biosensor representing ERK activity. JCam-LckWT/-LckYF/-LckKR and Jurkat cells expressing ERK biosensors were monitored before and after antibody stimulation. Before stimulation, JCam-LckWT and

Jurkat cells had higher basal FRET/ECFP ratio than the other groups (Fig. 4, A and D, and movie S2). After stimulation, the quantified FRET/ECFP ratio in JCam-LckWT, JCam-LckYF, and Jurkat cells increased to a similar level within 10 min, which can then be partially

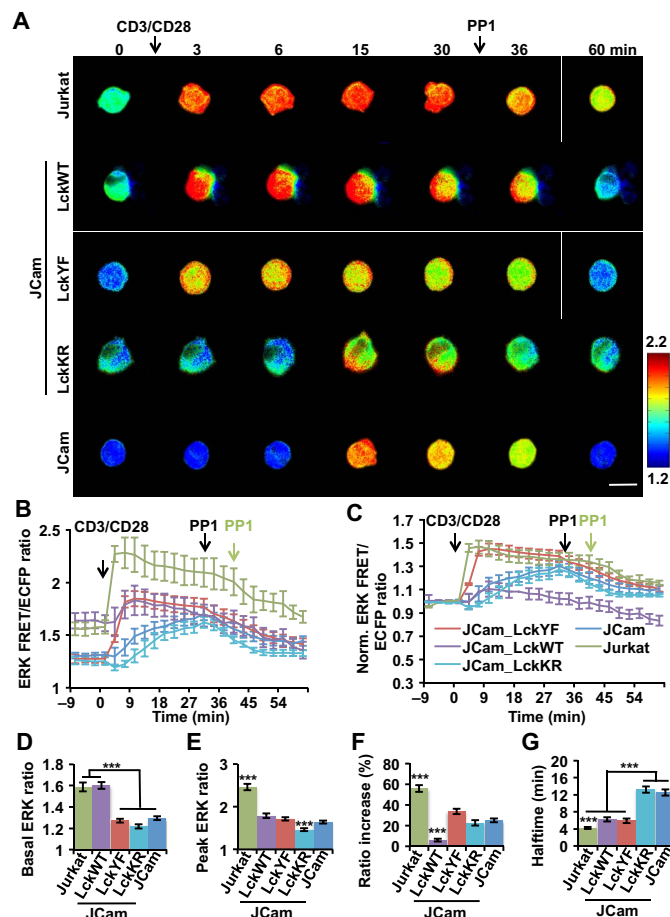


Fig. 4. Lck kinase activity mediates the fast activation of ERK kinase. The ERK biosensor was delivered by electroporation into Jurkat cells or JCam cells reconstituted with or without LckWT, LckY394F (LckYF), or Lck-K273R (LckKR) through lentivirus infection. Cells were stimulated with CD3/CD28 antibody clusters and then inhibited by PP1. (A) The FRET/ECFP ratio images of the ERK FRET biosensor in Jurkat and JCam cells before and after CD3/CD28 stimulation and PP1 inhibition. (B) Quantified FRET/ECFP ratio time courses (mean ± SEM) of the ERK biosensor in response to CD3/CD28 stimulation followed by PP1 inhibition as indicated. (C) Normalized FRET/ECFP ratio time courses of the ERK biosensor in T cells shown in (B). (D) Basal FRET/ECFP ratio level in the time courses shown in (B) before stimulation. Quantified basal ratio values are 1.59 ± 0.042 (Jurkat), 1.60 ± 0.033 (LckWT), 1.27 ± 0.017 (LckYF), 1.22 ± 0.021 (LckKR), and 1.29 ± 0.017 (JCam). (E) Peak FRET/ECFP ratio within 30 min after addition of CD3/CD28 antibodies in the time courses shown in (B). Quantified peak ratio values are 2.458 ± 0.077 (Jurkat), 1.785 ± 0.053 (LckWT), 1.713 ± 0.038 (LckYF), 1.463 ± 0.034 (LckKR), and 1.635 ± 0.034 (JCam). (F) Mean maximal increase of the FRET/ECFP ratio in the time courses shown in (C). Quantified ratio increased values are $55.88 \pm 3.60\%$ (Jurkat), $6.00 \pm 1.41\%$ (LckWT), $33.71 \pm 2.61\%$ (LckYF), $22.70 \pm 2.67\%$ (LckKR), and $24.96 \pm 1.95\%$ (JCam). (G) Half-time to reach peak ratio of T cells during 30 min after stimulation. The quantified half-time are 4.24 ± 0.23 (Jurkat), 6.26 ± 0.53 (LckWT), 5.89 ± 0.51 (LckYF), 13.22 ± 0.74 (LckKR), and 12.53 ± 0.72 (JCam). $N = 5$, $n = 62$, 119, 77, 41, and 83 for Jurkat, JCam with LckWT, LckY394F, LckKR, and JCam, respectively. ***Statistical difference from all other groups with $P < 1 \times 10^{-3}$. Error bars: mean ± SEM. Scale bar, 5 μ m. Representative Jurkat and JCam cells with or without Lck mutants are also shown in movie S2.

inhibited by PP1 (Fig. 4, fig. S3B, and movie S2). These results indicate that LckY394F can transduce TCR signals downstream to ERK activity with similar efficiency as that of LckWT.

Unexpectedly, the normalized FRET/ECFP ratio curves show that ERK can also be activated in the Lck-deficient JCam and JCam-LckKR cells (Fig. 4, C and F). Further analysis of the ERK activation kinetics showed that the ERK kinase was activated significantly slower in cells without functional Lck kinases than those with LckWT or LckY394F (Fig. 4, C and G), suggesting the role of the Lck kinase in mediating the fast ERK response upon activation. When the cells were treated with PP1, ERK activity decreased close to basal level in all groups, indicating that the observed slow ERK activation in Lck kinase-deficient cells may be mediated by an SFK member other than Lck (Fig. 4, B and C). In addition, when treated with PP1, the observed ERK kinase activity in JCam-LckWT dropped below the basal level. Accordingly, 61% of the ERK kinase was estimated to be preactivated in JCam-LckWT cells (Fig. 4, B and C). We further examined the level of endogenous phosphor-ERK in activated Jurkat and JCam cells with different Lck mutants (fig. S6). Western blot results showed that within 5 min of CD3/CD28 activation, significantly more phosphor-ERK can be detected in Jurkat, JCam-LckWT, and JCam-LckYF cells, but not in the JCam-LckKR cells (fig. S6). Therefore, we conclude that downstream of CD3/CD28 activation, the ERK kinase can be activated in both Lck kinase-dependent and Lck kinase-independent pathways, with Lck or LckY394F kinase mediating a fast ERK activation, while an SFK member other than Lck contributes to the slow phase of ERK activation. Lck activity is important for the rapid activation of downstream ERK signals, but the role of preactivated Lck seems unclear here (Fig. 4G). Overall, these results also verify that LckY394F upon stimulation has similar functionality as LckWT in mediating the activation of downstream T cell signaling.

CD3 is primarily responsible for the LckYF activation facilitated by CD28 costimulation

The high response level of JCam-LckYF cells allows us to explore the concentration-dependent kinetics of costimulation signals, as well as delineate roles played by CD3 and CD28 ligations. We, hence, stimulated the JCam-LckYF cells with different concentrations of combined CD3/CD28, CD3-only, or CD28-only antibody clusters while monitoring the Lck kinase activation dynamics with the ZapLck biosensor. Since multiple antibody combinations and concentrations were involved in these experiments, for simplicity, we defined the standard concentration of antibodies used in Figs. 2 to 4 as “1 unit” (10 and 5 $\mu\text{g}/\text{ml}$ for CD3 and CD28 antibodies, respectively; see Fig. 5). As a result, the lower concentration of “1/10 unit” corresponds to 1 $\mu\text{g}/\text{ml}$ and 0.5 $\mu\text{g}/\text{ml}$ for CD3 and CD28 antibodies, respectively, while “1/50 unit” represents 0.2 $\mu\text{g}/\text{ml}$ and 0.1 $\mu\text{g}/\text{ml}$ for CD3 and CD28 antibodies, respectively. All the antibodies were premixed and preclustered before treating cells as detailed in Materials and Methods.

As shown in Fig. 5 (A and B) and fig. S7, the quantified time courses of Lck activation show that different concentrations of CD3/CD28 antibodies or CD3-only antibody can all activate the Lck kinase with an average response of about 30% from the basal level. The magnitude of Lck kinase activation (normalized peak ratio) was relatively independent of the concentrations of combined CD3/CD28 antibodies, while the CD3-only groups showed significantly lower magnitude and slower kinetics at 1/50 unit (Fig. 5, A to C). The CD28-only groups did not show detectable Lck activation even at a high level of antibody treatment at 1 unit (fig. S8).

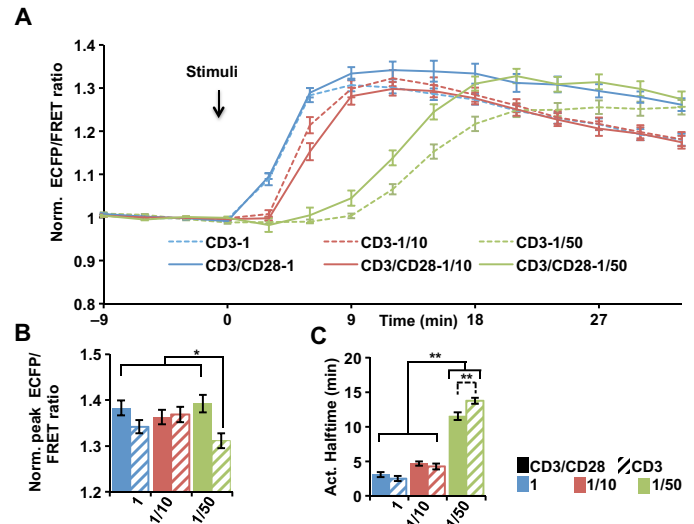


Fig. 5. The effect of CD3/CD28 co-receptor concentrations and CD28 on the dynamic kinetics of Lck activation. (A) Quantified ECFP/FRET ratio time courses (mean $\pm$ SEM) of the Lck biosensor in live JCam cells expressing LckYF treated with CD3/CD28 or CD3 only at different concentrations as indicated. Time courses were normalized such that the average ratio values before stimulation are 1. The labeling of CD3 and CD3/CD28 concentration conditions are defined in the Results section. (B) Comparison of the normalized peak ECFP/FRET ratio values of the Lck biosensors in 30 min after addition of antibodies in cells shown in (A). Quantified peak ratio values are 1.319 ± 0.019 ($n = 75$, 1 unit), 1.276 ± 0.017 ($n = 63$, 1/10 unit), and 1.281 ± 0.022 ($n = 69$, 1/50 unit) in the CD3/CD28 group, and 1.269 ± 0.018 ($n = 76$, 1 unit), 1.286 ± 0.018 ($n = 62$, 1/10 unit), and 1.197 ± 0.016 ($n = 64$, 1/50 unit) in the CD3 group. $N = 4$. (C) Half-time to reach peak ratio of T cells during 30 min after stimulation. Quantified peak ratio values are 3.082 ± 0.374 (1 unit), 4.665 ± 0.307 (1/10 unit), and 11.517 ± 0.581 (1/50 unit) in the CD3/CD28 group, and 2.486 ± 0.366 (1 unit), 4.253 ± 0.433 (1/10 unit), and 13.749 ± 0.452 (1/50 unit) in the CD3 group. Error bars: mean $\pm$ SEM. * and ** indicate statistical difference with $P < 0.05$, 0.01, respectively, from all other groups as indicated. Wilcoxon rank sum test was used without the need of assuming Gaussian distribution of data. The P values were adjusted to the comparison of multiple groups by summing the P values from individual tests with a result of significant difference.

Several measurements were calculated from the single-cell time courses to evaluate the activation kinetics and stability of Lck kinase signals (fig. S7). The activation half-time was calculated as the time gap between stimulation to when FRET ratio reaches half of its maximum in single cells, which is used to evaluate the activation speed of cellular Lck activation (Fig. 5C). Our results show that antibody concentration has a significant effect on the activation speed of the Lck kinase. The activation half-time almost tripled when the concentration was reduced from 1 to 1/50 for both CD3/CD28 and CD3-only antibody cluster stimuli (Fig. 5C). Judging from the time courses, the longer activation half-time is caused by both delayed response and slow activation kinetics at low-concentration stimuli (Fig. 5 and fig. S6). Furthermore, removing CD28 antibodies from the stimuli increased the activation half-time only at the lowest concentration of 1/50 (Fig. 5C). Together, our results indicate that CD3 is essential, while CD28 is supplementary, for Lck activation in T cells. This effect of CD28 on activation magnitude and speed only becomes prominent at the lowest antibody concentration of 1/50 (Fig. 5). Our results also show that antibody concentration mainly regulates the activation speed, but not the magnitude, of Lck kinase signals.

The kinase activities of LckWT and LckY394F are distinctively regulated at the basal level

We then asked why LckY394F has a basal activity distinctive from that of LckWT but both can reach a similar level in activities upon stimulation. Previous studies reported that Lck is preactivated and forms clusters in live T cells (26, 44). Our results also confirmed that LckWT, but not LckY394F, was preactivated in Jurkat and when expressed in JCam cells. Therefore, we hypothesize that the kinase activities of LckWT and LckY394F may be differentially regulated by their clustering or Lck-Lck interaction in T cells. Hypothesizing Lck expression levels and hence their surface densities may affect Lck-Lck interaction,

we investigated the effect of the expression level of Lck on its basal activity. Considering that Lck localizes to plasma membrane through its N terminus, we fused mCherry to the C terminus of Lck to obtain an Lck-mCherry construct, such that the expression level of Lck is represented by the mCherry fluorescence intensity. JCam cells expressing LckY394F-mCherry or LckWT-mCherry were cotransfected with the ZapLck biosensor to monitor the basal Lck activities by the FRET signals.

By quantifying the basal-level ECFP/FRET emission ratio versus mCherry intensity in the same single cells, we modeled the relation between the biosensor emission ratio (reflecting Lck kinase activity) and

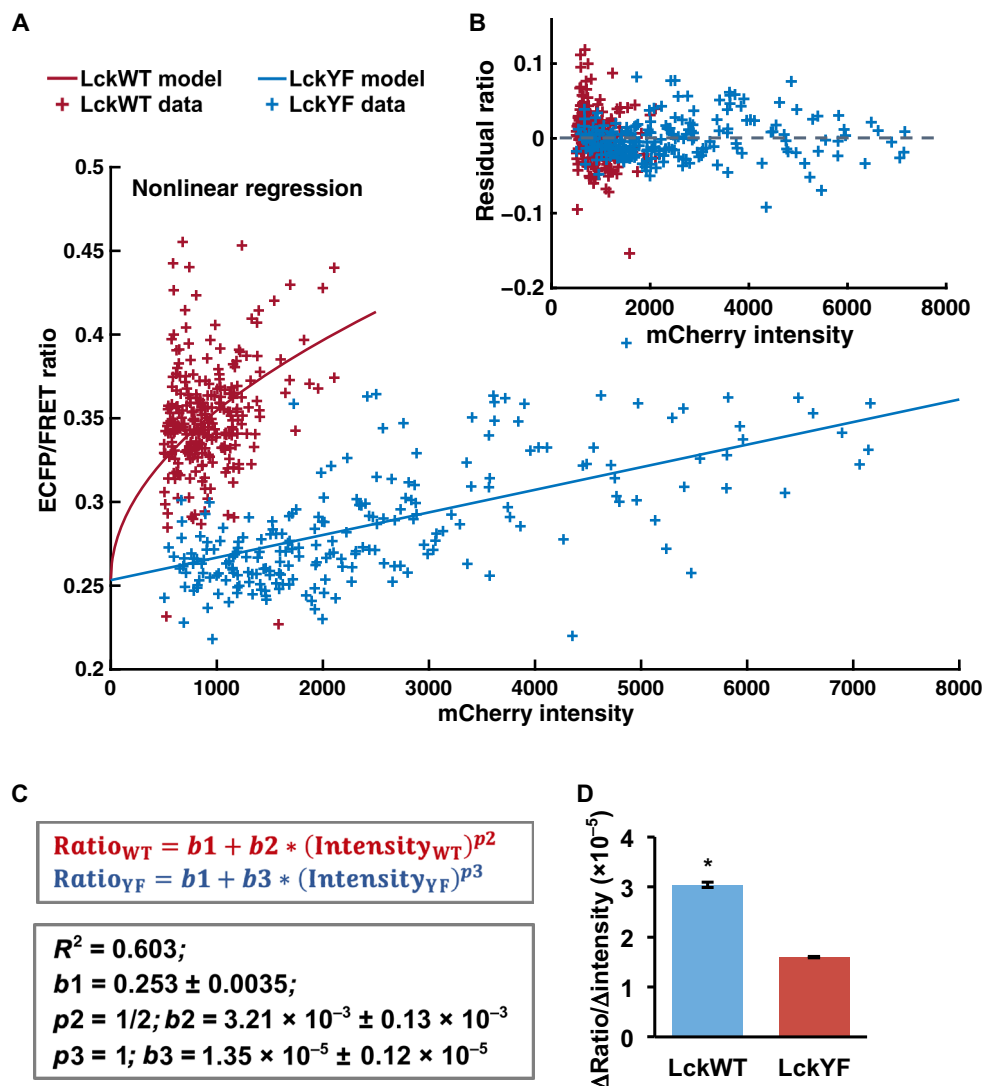


Fig. 6. Expression levels of LckWT and LckY394F distinctively affect their kinase activities. JCam cells were coinfecting by lentiviruses containing the ZapLck biosensor together with those with LckWT-mCherry (LckWT) or LckY394F-mCherry (LckYF). **(A)** Scatterplots show the basal FRET ratio of the biosensors versus the mCherry intensity in JCam cells. The LckWT data were fitted to a nonlinear model (red), and LckYF data were fitted to a linear model (blue). $N = 4$, $n = 243$, 207. Outlier cells were removed if their mCherry intensity deviates from the average value by three times SD or more. **(B)** Inset: Residuals of regression analysis from (A). **(C)** Mathematical models used for the nonlinear regression between LckWT mCherry Intensity ($\text{Intensity}_{\text{WT}}$) and ECFP/FRET Ratio (Ratio_{WT} , red), and for the linear regression between LckY394F mCherry Intensity ($\text{Intensity}_{\text{YF}}$) and ECFP/FRET Ratio (Ratio_{YF} , blue). Lower panel shows the R^2 values and fitted parameter (estimate $\pm$ SEM). Both nonlinear fitting and linear fitting were performed with MATLAB function NonLinearModel, with the constraint that both fitted curves intercept the ratio axis at the same height b_1 , which is the estimated basal ECFP/FRET ratio value of JCam cells without LckWT or LckY394F. **(D)** Quantification of the ratio between Δ ECFP/FRET and Δ mCherry intensity in JCam-LckWT ($n = 50$) and JCam-LckYF cells ($n = 42$). $N = 4$. The quantified $\Delta \text{Ratio} / \Delta \text{mCherry}$ intensity values are 3.042 ± 0.059 (LckWT) and 1.593 ± 0.012 (LckYF). *Statistically significant difference $P < 0.05$.

the mCherry intensity (reflecting the copy number of Lck or LckYF) in JCam cells expressing LckWT or LckY394F. JCam-LckWT-mCherry cells showed much lower expression level than the JCam-LckYF-mCherry cells, indicating that the expression level of LckWT, but not that of LckY394F, is strictly regulated in T cells (Fig. 6). We assume that the JCam cells without Lck expression (mCherry fluorescence intensity, FI = 0) have a minimal ECFP/FRET ratio of the value b_1 . Accordingly, we formulate a coupled model between the biosensor emission ratio and mCherry intensity for both JCam-LckWT and JCam-LckYF cells (Fig. 6C). This model predicts a ratio value of b_1 at zero mCherry intensity for both cell groups. The results revealed a nonlinear relation between Ratio_{WT} and $\text{Intensity}_{\text{WT}}$ in JCam-LckWT cells (the exponential power is the best fit at $p_2 = \frac{1}{2}$) but a linear relation between Ratio_{YF} and $\text{Intensity}_{\text{YF}}$ in JCam-LckYF cells ($p_3 = 1$, with balanced residuals) (Fig. 6, A to C). Therefore, we conclude that the correlation between LckWT kinase activity and expression is nonlinear, featured by a relatively faster increase of activity as the expression level increases in low-Lck-expressing cells but slower in high-expressing cells. In contrast, the activity of LckYF increases linearly as its expression level increases. The overall LckWT activity is also twice more sensitive to the expression level than that of LckYF (Fig. 6D). In these experiments, cells were selected into low-intensity (bottom 20 percentile) and high-intensity (top 20 percentile) groups, and ordered by intensity values in each group. A pairwise $\Delta\text{Ratio}/\Delta\text{Intensity}$ value was then calculated for two consecutive cells in the groups to evaluate the sensitivity of Lck kinase activity to expression level. These results suggest that LckY394F activity is less sensitive to expression level. Potentially, such an outcome could be explained by decreased interactions between LckY394F monomers compared with those of LckWT. In contrast, LckWT had a high basal kinase activity, which is significantly and nonlinearly affected by the expression level, suggesting a possible role of Lck-Lck interaction mediated by tyrosine phosphorylation. Together, these results indicate that the basal kinase activities of LckY394F and LckWT are distinctively regulated by their expression level, possibly via different modes of intermolecular interaction.

We reasoned that the distinctive states of LckWT and LckY394F in interacting and forming complexes can lead to a difference in their diffusion rates. We, hence, evaluated the diffusion rate of LckWT and LckY394F in live T cells with the fluorescence recovery after photobleaching (FRAP) experiment and finite element model analysis established in our laboratory (47). JCam-LckWT-mCherry and JCam-LckYF-mCherry cells with or without CD3/CD28 stimulation were photobleached and monitored for mCherry recovery (Fig. 7A). The images during recovery were used as input for a finite element diffusion model, which allows the quantification of Lck diffusion rate in live cells, relatively independent of cell shape, photobleach pattern, and initial intracellular fluorescence distribution (47, 48). The estimated apparent diffusion rates of LckWT and LckY394F with and without activation lie in the range of 0.60 to 2.13 $\mu\text{m}^2/\text{s}$, which is consistent with previous reported diffusion rates of Src and free lipid (Fig. 7) (49, 50). We found that at the basal level, LckWT diffused about two times slower than LckY394F (Fig. 7), indicating that LckWT may be significantly more aggregated than LckY394F. After stimulation, however, LckWT and LckY394F both had similar slow diffusion rates and diffused significantly slower than their basal levels (Fig. 7D). LckWT and LckY394F may form large-scale molecular complexes after stimulation and reduce their mobility at the plasma membrane of T cells. The diffusion speed of LckWT and LckY394F appears to be inversely related to their respective kinase activity, indicating that the level of Lck-Lck interaction

may reflect and possibly regulate their kinase activities in live T cells (Figs. 3 and 8).

Since diffusion speed is an indicator of the interacting state of a molecule at the plasma membrane, we performed additional imaging experiments with LckWT and LckY394F tagged with bimolecular fluorescence complementation (BiFC) split Venus (the N-terminal VN173 and C-terminal VC155 fragment of Venus, a yellow fluorescence protein) to compare the interacting states of Lck with or without the Y394F mutation, and before and after CD3/CD28 stimulation (Fig. 8). Specifically, Lck-Lck interaction can cause the binding between VN173 and VC155, which completes the formation of Venus to produce fluorescence signals after about 50 min of chromophore maturation (Fig. 8A). The JCam cells expressing LckWT- or LckYF-split Venus were stimulated with CD3/CD28 antibody clusters. Imaging and quantification results showed that both group of cells had significant increase in Venus intensity after stimulation, with a lower background and larger increase of dimerization detected in LckY394F cells than in the LckWT group (Fig. 8, B and C). These results showed that CD3/CD28 activation can enhance dimerization of LckY394F and LckWT in JCam cells. Therefore, Lck-Lck interactions were positively correlated to kinase activities of LckWT and LckY394F in live T cells (Figs. 3 and 8).

Together, we conclude that the expression and interaction of Lck are tightly regulated in T cells. LckWT has a high level of aggregation, which may contribute to its kinase activity before CD3/CD28 stimulation. In contrast, LckY394F molecules remain relatively unbound and inactive at basal levels. Both LckWT and LckY394F may form complexes and become activated with slow diffusion rates following CD3/CD28 stimulation.

DISCUSSION

Single-chain substrate-based FRET biosensors have been widely used to provide real-time visualization of posttranslational modification dynamics in live cells (27, 28). With a fixed (1:1) donor-to-acceptor ratio, the signals from these biosensors can be conveniently quantified by taking the ratio between donor and acceptor intensity. Biosensors designed to monitor enzymes typically contain a targeting substrate, but not enzymatic domains, so they should not cause significant perturbation of endogenous signals when expressed in live cells. As a result, these biosensors can function as a “real-time immunostaining assay” in single live cells to provide accurate and dynamic readout on the phosphorylation status of a selected substrate peptide, rendering a powerful imaging tool complementary to immunostaining and biochemical assays, which either require the killing of cells or only measure the average signal of a population of cells. On the basis of the design principle for single-chain and substrate-based FRET sensors (Fig. 1A), our group has successfully engineered and characterized several biosensors to visualize the kinase activity of Src, FAK, and Zap70 (31, 32, 51), some of which use our highly sensitive FRET pair, ECFP and YPet, for enhanced dynamic range in signals. With a similar strategy, here we engineered the new Lck biosensor by rationally examining several known tyrosine substrate peptides of the Lck kinase, including those from Zap70, ITAM, and Lck itself. The optimized ZapLck biosensor contains a Zap70 tyrosine peptide with a single tyrosine-to-phenylalanine mutation, which is identified as the most sensitive and specific among all substrates, with about 40% increase of signals in activated T cells.

Using this new biosensor, we investigated the molecular mechanism underlying the Lck activation in live T cells. Some reports suggest that there may be just ~2% of Lck with phosphorylated Y394 in resting

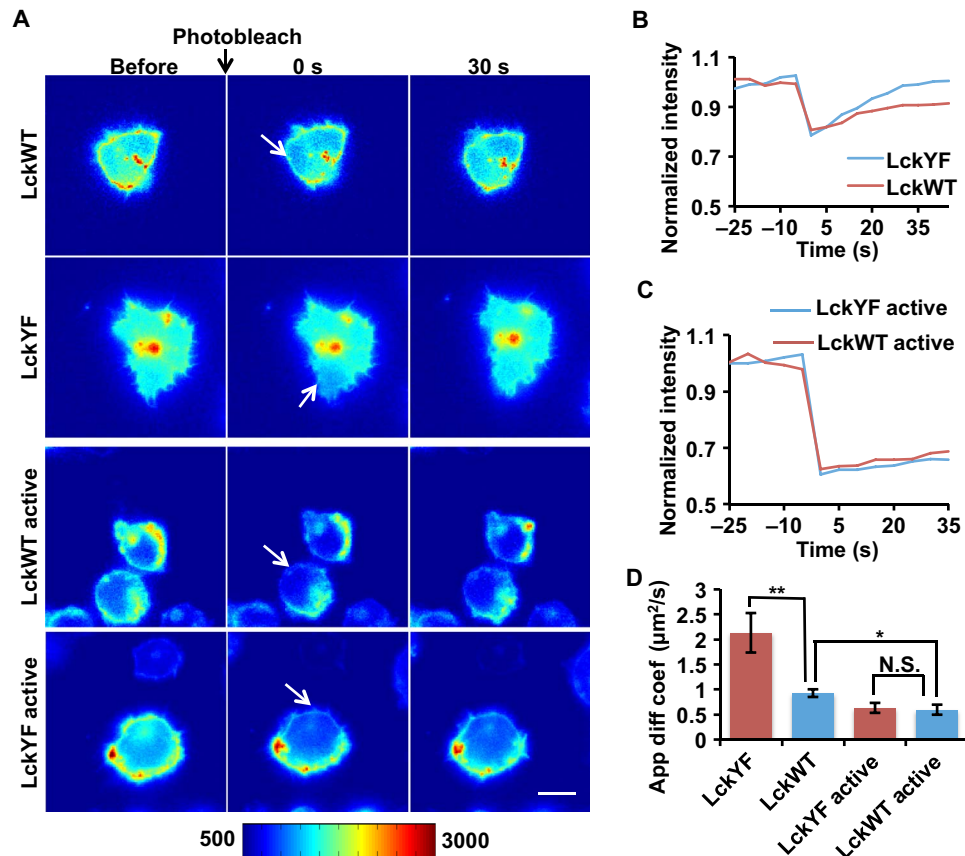


Fig. 7. Diffusion rates of LckWT and LckY394F in T cells. (A) Fluorescence intensity images of a representative cell before and after photobleaching in groups with (LckWT active and LckYF active) or without CD3/CD28 co-receptor activation (LckWT and LckYF), with the white arrows indicating the regions of interest monitored and quantified for fluorescence photobleach and recovery. (B and C) Time courses of fluorescence recovery in the photobleached area of representative cells in (A). (D) Bar graph shows the apparent diffusion coefficients (mean $\pm$ SEM) of LckWT and LckYF in JCam cells before (LckWT and LckYF) or after activation by CD3/CD28 antibody clusters (LckWT active and LckYF active). Quantified apparent diffusion coefficient values are 2.13 ± 0.4 (LckYF), 0.93 ± 0.08 (LckWT), 0.63 ± 0.1 (LckYF active), and $0.60 \pm 0.1 \mu\text{m}^2/\text{s}$ (LckWT active). * and ** represent statistically significant difference, $P < 0.05$ and $P < 0.01$, respectively and $N = 3$, $n = 23, 48, 12$, and 17 . Scale bar, $5 \mu\text{m}$. N.S., not significant.

T cells; however, other groups argue that there may be up to $\sim 40\%$ Lck phosphorylated on Y394 (44, 52). Phosphorylation of Y394 has been widely used as an assay to probe Lck kinase activity since there is a lack of Lck kinase reporters so far. Through signals reported by the ZapLck FRET biosensors, we found that up to 62 and 51% of Lck kinase were preactivated in Jurkat and JCam-LckWT cells, respectively, while LckY394F almost completely abolished the preactivation in JCam cells. As such, our results support the notion that a substantial fraction of Lck should be preactivated with phosphorylated Y394. It is possible that Lck preactivation is facilitated by the interaction between free Lck, ITAMs, and Zap70 (53, 54), with phosphorylated LckY394 serving as a docking residue. The mutation Y394F may affect the binding affinity and, hence, significantly reduce the basal-level Lck activation.

The overall Lck kinase activity is regulated by the phosphorylation dynamic equilibrium between the Y505 and Y394 tyrosine residues (55). The Y394F mutation has been reported to either completely abolish or decrease the Lck kinase activity to a very low level (21, 23, 26). However, our ZapLck biosensor visualized an increase of LckY394F kinase activity to be $\sim 92\%$ of LckWT activation upon CD3/CD28 ligation and clustering in live JCam cells (Fig. 3). Hence, our results suggest that LckY394F can be fully functional in its kinase activities upon CD3/CD28 clustering, possibly via enrichment and sequestration of CD3/CD28 and LckY394F

at the same submembrane compartments mediated by actin polymerization and raft formation (56). This kinase activation of LckY394F has not been observed in T cells previously, probably due to the lack of precise dynamic assays and methods in single T cells (21, 23, 26). The ZapLck biosensor reported a 40% increase of Lck kinase activity in JCam-LckYF cells upon co-receptor ligation. This dynamic range allowed us to investigate concentration dependence and individual contribution of co-receptors, and delineate the different roles played by CD3 and CD28 receptors. As such, these findings highlight the exquisite sensitivity of our ZapLck biosensor in detecting Lck kinase activity in single live T cells.

To further explore the effect of mutant LckY394F on downstream TCR signals, we used the ERK biosensor to monitor ERK kinase activity, which stimulates the expression of IL-2 and nuclear factor κB . We found that Lck kinase-dependent ERK activation responds faster to stimulation than ERK activation independent of Lck. Therefore, our data suggest that there are two signaling pathways controlling ERK activation: (i) fast Lck dependent and (ii) relatively slow Lck independent (5, 6). We also found that LckY394F can successfully activate ERK with kinetics similar to LckWT, verifying the functionality of LckY394F.

The diffusion speed of both LckY394F and LckWT are precisely correlated with their kinase activity in T cells before and after co-receptor

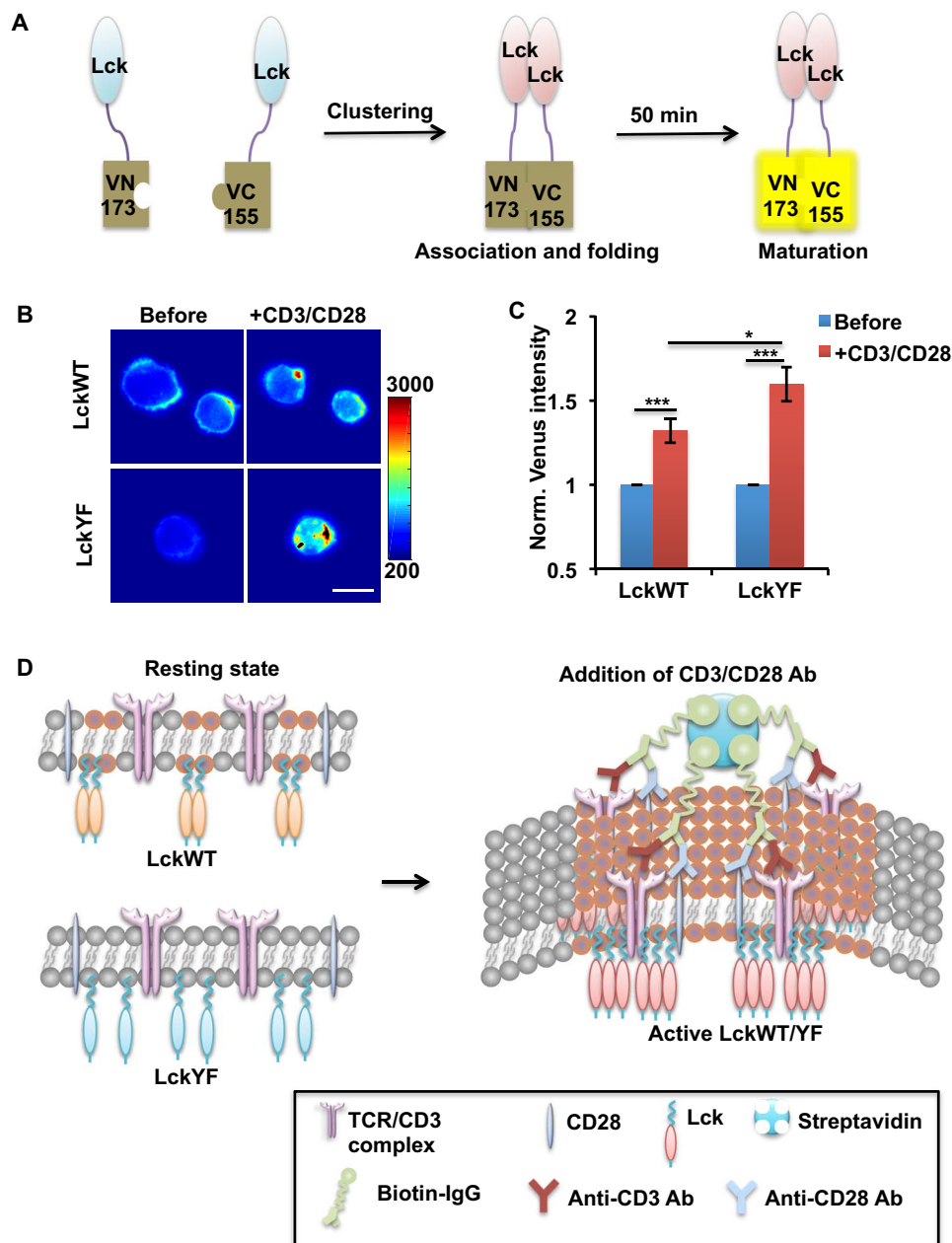


Fig. 8. The interaction and activation of LckWT and LckYF before and after CD3/CD28 stimulation. (A) Schematic drawing shows the principle of BiFC-split Venus in visualizing Lck-Lck interaction. (B) Fluorescence intensity images of representative cells before and after CD3/CD28 co-receptor activation. Scale bar, 5 μ m. (C) Bar graph shows the normalized Venus intensity of LckWT and LckYF in JCam cells before and after activation by CD3/CD28 antibody clusters for 3 hours. $N = 3$, $n = 147, 137, 118$, and 136 for LckWT-before, LckWT and CD3/CD28, LckYF-before, and LckYF and CD3/CD28, respectively. Error bars: mean $\pm$ SEM; * $P < 0.05$, *** $P < 1 \times 10^{-3}$. (D) Proposed mechanism of Lck interaction and activation. At the resting stage, the LckWT molecules form complexes via phosphorylated Y394 at T cell membrane in a preactivated state. Upon antigen binding, activated LckWT can be further activated and aggregated with the TCR/CD3 complex. The mutant LckYF (Y394F) molecules are relatively unbound and not preactivated, but they can be activated and aggregated similarly to LckWT by CD3/CD28 stimulation. Lck molecules were color coded by their kinase activity, with the cold and hot colors representing low and high activities, respectively. Ab, antibody.

activation (Fig. 7). LckY394F diffuses faster at the basal level when it is inactive, while basal LckWT, activated LckY394F, and LckWT all diffuse slower (Fig. 7). Besides diffusion speed as an indicator of the clustering state of a molecule at the plasma membrane, our experiments with Lck-split Venus further confirmed that both LckWT and LckY394F showed an enhanced dimerization and potential clustering after stimulation. These results suggest that the interaction and possibly clustering

status of Lck may reflect its kinase function and, in turn, regulate its kinase activities. Rossy *et al.* have suggested in 2013 an impaired clustering in the Y394F mutant in TCR-activated settings (25). Our study is different in that we used preclustered CD3/CD28 antibodies in the medium, but not coated on cover glass. Therefore, the enhanced Lck-Lck interaction for LckY394F in our study is prominent after activation, in contrast to the previous report in which the antibodies were fixed and

immobile on a solid surface. We were also able to visualize Lck kinase activity directly in live cells and connect Lck-Lck interaction with its ability to overcome the deficiency of Y394F to induce kinase activity.

It is possible that the phosphorylation of Y394 in LckWT enables a “sticky” property of the molecule, which allows intermolecular interactions, enhancing Lck clustering and enabling its preactivation even at low expression levels in T cells. This hypothesis is also consistent with reports that the open conformation of Lck can be stabilized by intact Y394, which can mediate the clustering of Lck during T cell activation (Fig. 6) (22, 25, 57), and that LckWT has a higher level of clustering than LckY394F (25). We hypothesized that Lck clustering can affect its kinase activity: Clustered Lck can potentially transphosphorylate a neighboring Lck at Y192 in the SH2 domain (57, 58) to reduce the inhibitory binding between the intramolecular Lck SH2 and pY505 (59), leading to the opening and activation of the neighboring Lck, and vice versa. In contrast, LckY394F is unable to cluster at the basal level, and its kinase activity is relatively low and linearly dependent on its expression (Fig. 6). TCR and its costimulation receptor clustering can fully enable the clustering and kinase activation of LckY394F (Fig. 8). As such, we conclude that a major role of Y394 within Lck is to provide a phosphorylatable anchoring motif mediating the Lck-Lck interaction and potentially clustering and maintaining a high basal activity in live cells.

In summary, we developed a new substrate-based Lck FRET biosensor with high sensitivity and spatiotemporal resolution to monitor Lck kinase activity in live T cells. Our data showed that the expression of LckWT was strictly controlled in T cells, where the molecules form complexes with slow diffusion and preactivated kinase activity at the basal state. These preactivated Lck population provides a fast and efficient mechanism to phosphorylate and activate TCRs upon stimulation as TCRs contain favorable substrate sequences for Lck (60). On the other hand, although the mutant LckY394F is relatively unbound and less preactivated with fast diffusion rates, CD3/CD28 clustering can trigger the formation of a submembrane compartment to cause the accumulation of CD3/CD28 and LckY394F at the same region for triggering complex formation and achieving activation to the level similar to that of LckWT (Fig. 8). Consequently, both LckWT and LckY394F can mediate fast downstream Lck-dependent ERK activation. These findings highlight the power of our FRET-based ZapLck biosensor in deciphering dynamic Lck regulation and function in TCR signals. Our findings can, hence, provide insight into the functional role played by the Lck kinase in T cell activation and potentially guide the optimization of engineered T cells for therapeutics.

MATERIALS AND METHODS

DNA constructs and virus production

Lck biosensors with different substrates were constructed using a published Src biosensor as the template (31), replacing the Src-specific substrate with Lck-specific substrates. For bacterial expression, the Lck biosensors were inserted into a pRSET-B backbone plasmid and transformed into BL21 competent *E. coli* cells for protein expression. For the mammalian cell expression, Lck biosensors were inserted into a customized pCAGGS vector using Gibson Assembly (New England BioLabs) (46). The biosensors were transformed into DH5 α competent *E. coli* cells for DNA amplification. LckWT and Lck mutants were inserted into a customized pCAGGS vector. The initial template constructs LckWT-CFP, LckK273R-CFP, and LckY394F-CFP were gifts from Dr. Katharina Gaus (Centre for Vascular Research and Australian Centre for NanoMedicine,

University of New South Wales, Sydney, Australia). For lentiviral production, fragments of the ZapLck biosensor, LckWT, LckY394F, and LckK273R, were amplified using polymerase chain reaction (PCR) and then inserted into a pSIN vector between Spe I and Eco RI restriction enzyme sites. For expression level experiments, LckWT and LckY394F were fused with mCherry at the C terminus through overlap PCR and inserted into the pSIN vector for lentiviral production. For visualization and analysis of Lck-Lck interaction, LckWT and LckY394F were fused with VN173 and VC155, respectively, at the C termini and inserted into the pSIN vector through Gibson Assembly. All the fragments were amplified using Q5 high-fidelity DNA polymerase (New England Biolabs, cat#M0491L). The pBiFC-bJunVN173 (cat#22012) and pBiFC-bFosVC155 (cat#22013) used as templates of VN173 and VC155 were purchased from Addgene.

To amplify the viruses, human embryonic kidney (HEK) 293T cells were cotransfected with the target plasmids contained in the pSIN vector, pCMV Δ R, and pCMV-VSVG using Lipofectamine 2000 (Invitrogen) in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS). Eight hours later, we changed the medium with advanced DMEM containing 2% FBS and 0.2% penicillin-streptomycin. After 48 hours, we collected the virus and purified the sample with a 0.22- μ m filter. To infect Jurkat or JCam cells, the virus was added to cells in a 1:1 volume ratio (with cell concentration below 1×10^6 cells/ml). For JCam cells infected with LckWT, LckY394F, or LckK273R, we used puromycin (1 μ g/ml) to select positive cells over a duration of 1 week before imaging.

In vitro kinase assay

The Lck biosensor plasmids were transformed into BL21 competent cells for protein amplification. Biosensor proteins were purified with the Kimble-Chase Protein Purification Kit (Fisher Scientific). For in vitro kinase assays, purified biosensor proteins were diluted to a final concentration of 1 μ M in kinase assay buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 2 mM dithiothreitol] containing 1 μ g/ml active Lck, Fyn, or Src kinases in a 96-well plate (100 μ l of total volume per well). The reaction was performed at 37°C for 3 hours. The emission spectra of the biosensor solutions were collected every 3 min with the plate reader (Tecan Infinite M1000 PRO using the i-control 1.10 software) before and after the addition of ATP (1 mM). The sample was excited at 427 nm, and the emission ratio (ECFP/FRET) was calculated according to the fluorescence intensity readouts at 476 and 528 nm for ECFP and FRET emission intensity, respectively.

Electroporation

Cells were passaged the day before electroporation. We washed about 5 to 10 million cells with PBS twice. Opti-MEM medium (500 μ l) was added to the resuspended cells. To deliver a single DNA plasmid, we added 15 μ g of plasmids to the resuspended cells. To introduce two or three plasmids, we added 10 μ g of DNA for each plasmid to the resuspended cells. We then transferred the mixture to the 4-mm cuvette (Bio-Rad) for electroporation (270 V, 950 μ F, infinite resistance) while avoiding bubbles. After electroporation, cells were transferred from the cuvette to the 35-mm dish with 5 ml of transfection medium for culture in the incubator.

Cell preparation for imaging

Jurkat and JCam cells were cultured in RPMI 1640 medium (Gibco) containing 10% FBS, 1% penicillin-streptomycin, 1% sodium pyruvate, and L-glutamine (300 mg/l). HeLa cells and HEK 293T cells were cultured

in DMEM (Gibco) containing 10% FBS, 1% penicillin-streptomycin, and L-glutamine (584 mg/l). All cells were incubated in a 5% CO₂ and 95% humidified incubator at 37°C. The cells were introduced with different biosensors and Lck variants via electroporation, transfection, or lentiviral infection depending on the cell types. Specifically, in Fig. 2 and fig. S2, transient transfection or electroporation was used. For Figs. 3 to 7 and figs. S3 to S8, lentiviral infection was used to introduce Lck mutants and Lck biosensors, while ERK biosensors were introduced by electroporation. In general, it is easier to deliver the genes into Jurkat cells and primary human T cells with lentiviral infection. On the basis of our experience, the performance of ratiometric FRET biosensor proteins is similar when expressed via lentiviral infection, lipotransfection, or electroporation.

HeLa cells were cultured in DMEM for 36 hours and then transferred onto glass-bottom dishes (Cell E&G) to be starved overnight before imaging. The dishes were coated with fibronectin (10 µg/ml) in PBS at 4°C overnight. During imaging, cells were stimulated with EGF (100 ng/ml). For Jurkat and JCam cells, the cells were seeded onto glass-bottom dishes (Cell E&G) and kept in the incubator for at least 10 min before imaging. In this case, the glass-bottom dishes were precoated with nonspecific immunoglobulin G (IgG) secondary antibody (10 µg/ml). During imaging, the cells were stimulated with CD3/CD28 antibody clusters. The CD3 antibody (10 µg/ml) and CD28 antibody (5 µg/ml) mixture was preclustered with IgG conjugated to biotin and then further coupled with streptavidin, kept at 4°C, before being used to stimulate the Jurkat and JCam cells. This concentration of CD3/CD28 antibody clusters was used as the baseline concentration of 1 unit (CD3 antibody at 10 µg/ml and CD28 antibody at 5 µg/ml), which was then diluted to 1/10 and 1/50 unit to compare the effect of concentration on Lck activation. In specified experiments, SFKs in cells were inhibited by 10 µM PP1 to observe the biosensor responses.

Human PBMCs were isolated from buffy coats (San Diego Blood Bank) using Ficoll gradients (Amersham Biosciences). CD3⁺ T cells were isolated from PBMCs using the Pan T Cell Isolation Kit (Miltenyi). For lentiviral transduction, cells were first activated for 72 hours using PHA (phytohemagglutinin) (Fisher Scientific, R30852801) in complete RPMI medium with IL-2 (100 IU/ml). The cells were then transduced with concentrated ZapLck biosensor lentivirus at a MOI (multiplicity of infection) of 10 by spinoculation on RetroNectin (Takara)-coated plates at 1800g, 32°C, for 1 hour.

Microscope imaging and analysis

For FRAP experiments, the mCherry images were collected using the MetaFluor 6.2 software (Molecular Devices, Sunnyvale, California) on an epifluorescence microscope (Olympus IX81) with excitation at 572DF35 and emission at 632DF60 using 1% of the light source power. During imaging, the cells were kept in RPMI 1640 medium without serum at 37°C, and the objective was focused at the bottom of the cell. The cells were monitored before photobleaching to confirm that the fluorescence signals were stable during imaging. Photobleaching was conducted by exciting mCherry at 560DF20 in a region of interest with full power of the light source for 15 s. Subsequently, the recovery process was imaged at 5-s intervals for the mCherry fluorescence signals (47).

For FRAP analysis, we used a finite element-based diffusion analysis method, which was previously developed and rigorously characterized by us to allow estimation of diffusion coefficients based on images collected by wide-field microscopes. The methods and results have been published in *PLOS Computational Biology* (47). With this method, the photobleach experiment protocol is simple and does not require the usage of a confocal microscope. The analysis method can be applied

to cells of complex geometry without restrictions on the photobleaching pattern and protocol. Only one fluorescence intensity image before photobleaching and two images after are required for estimating the diffusion coefficient without the need of a complete time course.

For FRET experiments, the images were collected with a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters controlled by a filter changer (480DF30 for CFP and 535DF25 for FRET). The pixel-wise images of the CFP/YFP emission ratio were computed to quantify the FRET signals, which represent the concentration of the phosphorylated Lck biosensor and, hence, Lck activity in space and time.

A Nikon Eclipse Ti inverted microscope installed with a 300-W Xenon lamp (Atlas Specialty Lighting), an electron multiplying (EM) charge-coupled device camera (QuantEM:512SC, Photometrics), and a 100× Nikon microscope objective (numerical aperture, 1.45) were used to capture all imaging data with the MetaMorph 7.8 software (Molecular Devices). Analysis of all acquired images was conducted on FluoCell, an image analysis software tool developed in the Wang Lab (unpublished results, S.L. at the University of California).

Western blot

The antibodies used for the immunoblot analysis are as follows: anti-perk(1/2) (4377T), anti-ERK(1/2) (9102S), and anti-glyceraldehyde phosphate dehydrogenase (2118S) from Cell Signaling Technology. 4G10 was from Millipore (05-321), and anti-GFP (ab290) was from Abcam. Briefly, the cells were stimulated as indicated, and then the cells were lysed using radioimmunoprecipitation assay buffer (9806S) on ice for 10 min and then centrifuged at 4°C and 13,000 rpm. The protein concentration was measured using a Bio-Rad protein assay dye reagent (Bio-Rad, #5000006) following the protocol of the manufacturer. The same amount of boiled proteins was loaded into precast 4 to 20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) (cat#4561096) or 10% precast SDS-PAGE gel for ERK phosphorylation measurement. The gel was run at 110v for 60 to 90 min and then transferred to a nitrocellulose filter membrane at 110v for 60 min. Dry milk (5%) was used to block the membrane at room temperature for 30 min. Then, the membrane was incubated with primary antibody at 4°C overnight, followed by incubation with the secondary antibody at room temperature for 1 hour. Last, the membrane was visualized with standard chemiluminescence.

Statistical analysis

Two-tailed Student's *t* tests with unequal variants or the Wilcoxon rank sum tests were used for statistical analysis. The *P* values were adjusted to the comparison of multiple groups either by the MATLAB multcompare function or by summing the *P* values from individual tests with a result of significant difference. The analysis methods were also described in the figure legends. *N* represents the number of independent experiments, and *n* represents the number of cells. All the results shown in the main figures had three or more independent repeats.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/5/6/eaau2001/DC1>

- Fig. S1. Further in vitro characterization of the Lck biosensors.
- Fig. S2. Characterization of Lck biosensors with different substrates in HeLa cells.
- Fig. S3. Characterization of the ZapLck biosensor in JCam cells.
- Fig. S4. Preactivation of Lck in Jurkat and PBMCs.
- Fig. S5. Characterize the subcellular localization of mCherry-tagged Lck mutants, and the sensitivity of the ZapLck biosensor in detecting Lck activity compared with a Src biosensor.

Fig. S6. Phosphorylation level of endogenous ERK in T cells stimulated by CD3/CD28 antibody clusters.

Fig. S7. Activation of Lck kinase in JCam cells with different stimuli at different concentrations. Fig. S8. CD28-only antibody did not trigger TCR signals.

Movie S1. The dynamic change of ZapLck biosensor ECFP/FRET ratio signals under CD3/CD28 stimulation and PP1 inhibition in Jurkat or JCam cells with or without Lck or Lck mutants as indicated.

Movie S2. The dynamic change of ERK (NES) biosensor FRET/ECFP ratio signals under CD3/CD28 stimulation and PP1 inhibition in Jurkat or JCam cells with or without Lck or Lck mutants as indicated.

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