**Articles**

**In vitro** membrane reconstitution of the T-cell receptor proximal signaling network

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T-cell receptor (TCR) phosphorylation is controlled by a complex network that includes Lck, a Src family kinase (SFK), the tyrosine phosphatase CD45 and the Lck-inhibitory kinase Csk. How these competing phosphorylation and dephosphorylation reactions are modulated to produce T-cell triggering is not fully understood. Here we reconstituted this signaling network using purified enzymes on liposomes, recapitulating the membrane environment in which they normally interact. We demonstrate that Lck's enzymatic activity can be regulated over an ~10-fold range by controlling its phosphorylation state. By varying kinase and phosphatase concentrations, we constructed phase diagrams that reveal ultrasensitivity in the transition from the quiescent to the phosphorylated state and demonstrate that co-clustering TCR and Lck or detaching Csk from the membrane can trigger TCR phosphorylation. Our results provide insight into the mechanism of TCR signaling as well as other signaling pathways involving SFKs.

Regulation of protein phosphorylation underlies many signal-transduction pathways that govern cellular processes. One well-studied signal-transduction cascade is the T-cell adaptive immune response, which is initiated by the interaction of the T cell receptor (TCR) with peptide-major histocompatibility complex (pMHC) on an antigen-presenting cell (APC). The immediate consequence of TCR-pMHC binding is the tyrosine phosphorylation of the immune receptor tyrosine-based activation motifs (ITAMs) of the TCR complex. The phospho-ITAMs recruit the cytosolic protein tyrosine kinase ZAP-70, which subsequently catalyzes additional phosphorylation reactions to turn on downstream signaling cascades.

Unlike many receptors that contain an intrinsic tyrosine kinase domain, the TCR itself lacks kinase activity but harbors ten ITAMs (20 potential tyrosine phosphorylation sites) on the CD3 subunits (ζζ, δε and εζ pairs). TCR phosphorylation is principally carried out by the SFK Lck and reversed by the transmembrane phosphatase CD45. Therefore, Lck and CD45 form a minimal network that controls the phosphorylation state of TCR. Similar to other members of SFKs, Lck is attached to the inner leaflet of the plasma membrane via N-terminal myristoylation and palmitoylation. SFKs are believed to be reciprocally regulated by phosphorylation and dephosphorylation of two conserved tyrosine residues¹: phosphorylation of a tyrosine (Y505) at the C-terminal tail by the inhibitory kinase C-terminal Src kinase (Csk)²,³ leads to inhibition owing to intramolecular interaction of its Src homology 2 (SH2) domain with the phosphotyrosine⁴,⁵ and autophosphorylation of the activation-loop tyrosine (Y394) is thought to enhance the kinase activity. CD45, a highly abundant transmembrane tyrosine phosphatase required for T-cell development and activation⁶, dephosphorylates both regulatory tyrosines of Lck⁷–⁹ as well as reversing the action of Lck by dephosphorylating the ITAMs of the TCR¹⁰.

Although this general qualitative model of the proximal TCR phosphorylation network is well established, a quantitative understanding of the reaction network is lacking. Studies of Lck regulation have been sparse and have reported different results⁸,¹¹, and CD45 phosphatase’s specificity for phosphorylated Lck (pY505 and pY394) and phosphorylated TCR has not been examined. Furthermore, the net effect of competing kinase-phosphatase reactions on substrate phosphorylation has not been examined *in vitro* for any SFK, nor have phosphorylation reaction kinetics been measured on membrane surfaces (their physiological environment). To develop a quantitative understanding and predictive mathematical models for TCR signaling¹², reinvestigation of Lck, Csk and CD45 regulation, substrate specificity and activity levels using homogeneous, purified proteins and kinetic enzymatic readouts is warranted¹³.

Here, we set out to reconstitute the TCR proximal signaling network consisting of Lck, Csk, CD45 and a TCR subunit (CD3ζ) onto the membranes of unilamellar liposomes and developed fluorescence readouts of phosphorylation. This reconstitution system has allowed us to control the two-dimensional (2D) concentration of each protein and build the network in a stepwise fashion with increasing complexity. Using purified Lck with distinct phosphorylation states, we have been able to probe the regulatory mechanism of Lck in its native membrane environment. We also have characterized individual enzymatic reactions in isolation as well as in combination to construct phase diagrams of the TCR-kinase-phosphatase network to understand how the system is maintained at a quiescent state and how it might be activated by TCR ligation.

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RESULTS
Membrane reconstitution of Lck phosphorylation of CD3ζ

In order to study Lck catalyzed phosphorylation of ITAMs in a membrane environment, we first reconstituted Lck and its substrate CD3ζ onto artificial lipid bilayers. Lck is anchored to the inner leaflet of the plasma membrane through myristoylation of a conserved N-terminal glycine. We replaced the N-terminal glycine with a decahistidine (His10) tag so that Lck could be bound to liposomes containing DGS-NTA-Ni (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]) lipids (see Online Methods). An N-terminal His10 tag was similarly placed on the cytosolic domain of recombinant CD3ζ. Using a fluorescence resonance energy transfer (FRET)-based assay, we found that His10-tagged proteins stably bind to these liposomes with a K_d of 0.6 nM and an off-rate of 0.0009 s^{-1} (Supplementary Fig. 1a,b), in agreement with a previous study. To monitor the phosphorylation state of CD3ζ, we generated a fluorescence-labeled, SNAP-tag fusion protein of tandem SH2 domains (tSH2) of ZAP-70 (designated as SNAP505-tSH2) that binds doubly phosphorylated ITAMs on CD3ζ (Fig. 1a). Upon addition of ATP, phosphorylation of CD3ζ by Lck leads to recruitment of SNAP505-tSH2 to liposomes, as detected by FRET between SNAP505-tSH2 (donor) and rhodamine-conjugated lipids (acceptor) (Fig. 1b; decrease in donor fluorescence). Immunoblots revealed a similar time-dependent phosphorylation of CD3ζ (Fig. 1c), indicating that the FRET assay faithfully reports the phosphorylation of CD3ζ and that the presence of SNAP505-tSH2 does not interfere with the rate of the overall reaction.

Mathematical attachment accelerates Lck phosphorylation of CD3ζ

Mathematical modeling predicts that bimolecular reaction rates are much greater on membrane surfaces than in solution, as the diffusion search is confined to two dimensions. Indeed, Ras activation by Son of sevenless (SOS) was found to occur 500-fold faster on artificial liposomes than in solution. To compare the kinetics of Lck-catalyzed CD3ζ phosphorylation on membranes to that in solution, we measured FRET between tetramethylrhodamine bound to CD3ζ and SNAP505-tSH2 (see Online Methods). Similarly, we found that prebinding both Lck and CD3ζ to liposomes at their physiological densities (300 molecules per μm^2) led to phosphorylation rates that were several-hundred-fold faster than in solution and that the phosphorylation rate showed a positive correlation with the protein densities (Fig. 1d).

Recently, it was reported that acidic phospholipids bind to CD3 chains in resting T cells, thereby limiting the access of Lck to ITAMs. In contrast, we found here that CD3ζ phosphorylation occurred 1.8-fold faster on membranes with 10% phosphatidylycerine (PS; an acidic phospholipid) than on those with no PS; 20% PS did not further increase the rate (Supplementary Fig. 1e). We note that previous studies used the soluble cytosolic portion of CD3, whereas we pre-anchored CD3ζ chains to the membrane, mimicking the situation in cells. For the liposome assays in this study, we used 10% PS (see Online Methods), which is close to the concentration in the plasma membrane inner leaflet of human T cells (7.4% PS).

Lck autophosphorylates Y394 and Y505 in trans

The phosphorylation of two conserved tyrosines in SFKs has distinct roles in kinase regulation. Phosphorylation of Lck Y505, near the
Lck undergoes autophosphorylation on Y394 and Y505. (a) Western blot (WB) analysis of Lck autophosphorylation on membranes versus in solution. Left, kinetics of Lck phosphorylation at Y394 and Y505 upon ATP addition, with Lck either in solution (86 nM) or attached on liposomes (~500 Lck µm⁻², see Online Methods). Right, quantification of the immunoblots. Optical density (OD) for each band quantified, normalized to the last time point (180 min) under each condition, and plotted against time. (b) Immunoblots for measuring the ATP $K_M$ of Lck autophosphorylation at Y394 and Y505 (Lck density, 500 µm⁻²). The normalized WB signals at 5 min after ATP addition was plotted against ATP concentration and fit using Michaelis-Menten model, yielding $K_M$ values. (c) Time course of ATP-triggered phosphorylation of WT and kinase-dead Lck (K273R), when both proteins were attached to the same membrane. Left, cartoon showing the two proteins of interest reconstituted at the same density. The 13-kDa FKBP (inserted between His₁₀ and Lck) was introduced for electrophoretic separation of WT from kinase-dead Lck. Middle, immunoblot (top) showing the kinetics of Y505 phosphorylation upon ATP addition and the quantification plot (bottom). Right, Immunoblot (top) showing the kinetics of Y394 phosphorylation upon ATP addition and the quantification plot (bottom). For all immunoblots shown, samples were derived from the same experiment and the blots were processed in parallel. Original images of blots are shown in Supplementary Figure 9. pY394-Lck, phosphorylated Y394 in Lck; pY505-Lck, phosphorylated Y505 in Lck.

C terminus, leads to its intramolecular binding to the SH2 domain, resulting in a closed, inactive conformation of the kinase. In contrast, phosphorylation of Y394, in the activation loop, leads to kinase activation. It is generally believed that Y394 is autophosphorylated in trans, whereas Y505 phosphorylation is catalyzed by Csk, a negative regulatory kinase. Whether this is the sole mechanism for Y505 phosphorylation is unclear, with some prior studies suggesting that cellular-Src (c-Src) is capable of autophosphorylating Y527 (refs. 24, 25).

To clarify its mechanism of autophosphorylation, we incubated Lck with ATP and measured the phosphorylation of Y394 and Y505 over time with phospho-specific antibodies. The rate of Y394 phosphorylation was at least 100-fold faster on membranes (t₁/₂ < 0.25 and > 10 min on membranes and in solution, respectively, Fig. 2a). The massive rate increase upon membrane attachment suggests that Lck phosphorylates Y394 in trans.

We also observed a time-dependent increase in Y505 phosphorylation (Fig. 2a), indicating autophosphorylation on this tyrosine. The $K_{M,ATP}$ for Y505 phosphorylation (10 µM) was 45-fold higher than Y394 phosphorylation (0.22 µM). Interestingly, Y394F mutation further increased the $K_{M,ATP}$ for Y505 phosphorylation by five-fold (50 µM) (Fig. 2b), indicating that phosphorylation of Y394 increases the ATP sensitivity for subsequent Y505 phosphorylation (see Supplementary Fig. 2a–c for the time course of autophosphorylation at four ATP concentrations). The much higher $K_{M,ATP}$ for Y505 phosphorylation may explain why it was undetected in previous in vitro studies; however, the in vivo ATP concentration (1–10 mM) is more than sufficient for this phosphorylation to occur.

Y505 phosphorylation was only modestly increased by anchoring Lck onto liposomes (Fig. 2a), an observation that might suggest an intramolecular phosphorylation. To test whether Lck autophosphorylates Y505 in cis, we mixed equal concentrations of wild-type (WT) Lck and a kinase-dead (K273R) Lck mutant on liposomes and examined their autophosphorylation kinetics (Fig. 2c). In this mixture, the phosphorylation of Y394 and Y505 on K273R can occur only in trans. We observed that phosphorylation of both Y505 and Y394 occurred at indistinguishable rates for the two versions of Lck (Fig. 2c, right). From this experiment, we conclude that Lck autophosphorylates both Y394 and Y505 in trans.

Quantification of the magnitude of phosphoregulation of Lck
To determine the effects of phosphorylation on Lck kinase activity, a comparison of monomeric Lck with distinct, well-defined phosphorylation states is required. To this end, we have measured the enzyme kinetics of membrane-attached Lck in the unphosphorylated (designated as apo, prepared by CD45 treatment; Supplementary Fig. 2d and Online Methods), monophosphorylated (pY394 or pY505), and doubly phosphorylated (pY394-pY505) states. We prepared doubly phosphorylated Lck by incubating purified Lck with ATP until both Y505 and Y394 reached a maximum level (Online Methods and Supplementary Fig. 2e,f). MS revealed 82% and 97% phosphorylation on Y394 and Y505, respectively (Supplementary Fig. 3), suggesting that at least 79–82% Lck was doubly phosphorylated. Monophosphorylated Lck was similarly produced by autophosphorylation, with phosphorylation at one site blocked by a Y394F or a Y505F mutation. We measured the phosphorylation kinetics of CD3ζ by FRET over a wide range of substrate concentrations and
plotted the initial rates (v0) of phosphorylation as a function of CD3ζ concentration (Supplementary Fig. 4). Data were fit with a Hill-equation-modified Michaelis-Menten model (Fig. 3a,b), yielding kcat, KM and the Hill coefficients (nH) (Table 1). The observed kcat values varied from 1 s⁻¹ to 7 s⁻¹, in the same range as solution measurements on recombinant WT Lck using a peptide substrate corresponding to the autophosphorylation site of c-Src 26 but orders of magnitude higher than a recent study using peptide substrates corresponding to individual tyrosines in CD3ζ (ref. 27). The apparent 2D KM values (200–500 μm⁻²) are similar to physiological densities of CD3ζ (100–360 μm⁻²), suggesting that Lck is operating in T cells at close to its maximal capacity while maintaining sensitivity to changes in the substrate concentration.

Our analysis of the catalytic activity (kcat / KM) shows that tyrosine mutations themselves do not alter kinase activity, as the Y394F-Y505F double mutant and apo Lck showed similar catalytic activities for CD3ζ (see Supplementary Note for comparison with prior findings of these mutants). However, pre-phosphorylation of Y394 (in the Y505F background) enhanced the catalytic activity by 70%; in contrast, phosphorylation of Y505 (in the Y394F background) decreased the catalytic activity by 83%. Interestingly, when both tyrosines were phosphorylated, the catalytic activity returned to the apo kinase level, suggesting that the inhibitory effect of Y505 can be overcome by Y394 phosphorylation, in agreement with a recent study using immuno-precipitated Lck 28.

CD3ζ phosphorylation is largely nonprocessive.

c-Src was reported to catalyze the multisite phosphorylation of a synthetic peptide 29 or p130 Crk-associated substrate (p130Cas) 30 through a processive mechanism. Because CD3ζ contains six phosphorylatable tyrosines, we wished to determine whether Lck carries out multisite phosphorylation in a processive or nonprocessive (distributive) manner. Phosphorylation of CD3ζ resulted in slower electrophoretic mobility 31, enabling identification of intermediates between the nonphosphorylated and fully phosphorylated state. After addition of ATP to membrane-attached CD3ζ and Lck, we observed a time-dependent, upward shift of the CD3ζ band with clear intermediates (Fig. 3c). This result argues against a processive mechanism, which often leads to an abrupt transition between the apo and fully phosphorylated species 32.

We also examined the rate of formation of the fully phosphorylated product at different substrate concentrations. A processive kinase remains bound to the substrate, phosphorylating all the sites in one encounter; as a result, the onset of the fully phosphorylated substrate is independent of the substrate concentration. Conversely, in a distributive mechanism in which the formation of the fully phosphorylated product requires repetitive binding interactions between kinase and the same substrate, the encounter of a kinase with a partially phosphorylated substrate is decreased at higher substrate concentration due to peer competition. We found that increasing the CD3ζ concentration by three-fold considerably delayed the formation of the fully phosphorylated product (Fig. 3c), consistent with a nonprocessive (or distributive) mechanism. The processivity of Src has been attributed to its SH2 domain, which can potentially bind, in trans, to

Table 1 2D enzyme-kinetic parameters of Lck for distinct phosphorylation states

| Proteins                  | KM (μm⁻²) | kcat (s⁻¹) | kcat / KM (x 10⁻² μm⁻² s⁻¹) | nH  |
|---------------------------|-----------|------------|-----------------------------|-----|
| Lck (Apo)                 | 245 ± 32  | 3.41 ± 0.35| 1.39                        | 2.3 ± 0.5 |
| Lck (Y394F, Y505F)        | 234 ± 19  | 3.76 ± 0.27| 1.61                        | 2.9 ± 0.6 |
| Lck pY505 (Y394F)         | 428 ± 69  | 1.00 ± 0.15| 0.23                        | 2.2 ± 0.4 |
| Lck pY394 (Y505F)         | 272 ± 65  | 6.26 ± 0.55| 2.30                        | 2.9 ± 0.6 |
| Lck pY394-pY505           | 232 ± 19  | 3.29 ± 0.24| 1.42                        | 3.0 ± 0.6 |

kcat, KM and nH are presented as mean ± s.e.m., n = 4.
the phosphotyrosines in the partially phosphorylated substrate. However, we find that a SH2 deletion (ΔSH2) mutant of Lck produces a diffusive pattern of the phosphorylation products similarly to the WT kinase, though with slightly faster kinetics (Fig. 3d). Collectively, these data suggest that Lck phosphorylates CD3ζ in a largely nonprocessive manner, although we cannot rule out processive phosphorylation for the intact, multi-subunit TCR.

**CD45 enzymatic activity**

Although both Lck and CD3ζ are known substrates for CD45, the relative substrate specificity has not been determined in a quantitative manner. Using a recombinant cytoplasmic portion of CD45, we probed the time course of dephosphorylation of Y394 and Y505 in Lck and CD3ζ on liposome membranes. CD3ζ was dephosphorylated >20-fold faster than Lck by CD45 (Fig. 4a,b), indicating that it is a much better substrate. Y394 and Y505 of Lck were dephosphorylated at similar rates. Notably, the presence of tSH2 of ZAP-70 dramatically reduced the kinetics of CD45-mediated CD3ζ de phosphorylation (Fig. 4a,c), indicating that phospho-ITAMs can be protected from CD45 by bound ZAP-70.

**Phase diagrams of the Lck-CD45-CD3ζ network**

Understanding how the combined actions of kinases and phosphatases affect the output of signaling pathways is nontrivial, owing to the presence of the many regulatory reactions and the lack of knowledge of reaction rate constants (Fig. 5a). Here, we determined the combinatorial effects of Lck (WT or phosphorylation mutants) and CD45 on the phosphorylation of TCR on a membrane by mapping the phase diagram of the network (Fig. 5a). In this experiment, the substrate CD3ζ was kept at a fixed density (~400 molecules per µm²; see Online Methods), whereas the concentrations of both Lck and CD45 were varied 2–3 orders of magnitude (84 conditions). The level of CD3ζ phosphorylation at 1 h was probed by FRET (Fig. 1a), plotted against Lck and CD45 surface densities and converted into a heat map (Fig. 5b,c). A steady state was not reached in all conditions at 1 h (Fig. 5b), and end-point data at 0.5 h, 1.5 h and 2 h produced slightly shifted heat maps (Supplementary Fig. 5a–d) but did not change the general conclusions. At equal Lck and CD45 densities, CD3ζ phosphorylation remained strong. Increasing the Lck density enhanced the extent of CD3ζ phosphorylation, whereas increasing the CD45 density did the opposite, suggesting the predominant effect of CD45 on CD3ζ phosphorylation is inhibitory. At the physiological relevant regime, little to no phosphorylation of CD3ζ was observed. Mutation of the activating tyrosine (Y394F) shrank the phosphorylation-positive region considerably (Fig. 5c, middle). Conversely, the constitutively active Lck mutant (Y505F) expanded the phosphorylation positive region, indicative of a higher kinase activity (Fig. 5c, right).

These differences reflect the contribution of Lck autophosphorylation on the phase behavior of the network.

To determine whether there is an ultrasensitive concentration dependence, we plotted CD3ζ phosphorylation as a function of WT Lck to CD45 molar ratios (Lck/CD45, Fig. 5d), as described for other competing kinase-phosphatase reactions. The nH of this plot (~2.2) suggests a switch-like behavior. The half maximal CD3ζ phosphorylation for WT Lck occurs at a ratio of 0.68. We also examined the CD3ζ phosphorylation as a function of varying Lck or CD45 concentration, keeping the opposing enzyme at a fixed concentration (Fig. 5e,f). These differences reflect the contribution of Lck autophosphorylation on the phase behavior of the network.

**Regulation by the inhibitory kinase Csk**

Csk has an important negative regulatory role in signaling by phosphorylating Lck on Y505 (ref. 23). Membrane-bound Csk (~500 µm²) accelerated Y505 phosphorylation by ~12-fold, but had little effect on the rate of Y394 phosphorylation (Fig. 6a). Reducing the Lck...
density to ~50 μm⁻² in the presence of Csk (~500 μm⁻²) only slightly accelerated the phosphorylation of Y505, but substantially retarded Y394 phosphorylation (Fig. 6b). We also repeated the experiments in solution and confirmed the finding that Csk not only promotes the phosphorylation of Y505, but also inhibits the phosphorylation of Y394 (Supplementary Fig. 6). We propose that a lower ratio of Lck to Csk kinetically favors initial Y505 phosphorylation, which would inactivate a subpopulation of Lck and, in turn, decrease the kinetics of trans-autophosphorylation on Y394 by reducing the active kinase on the membrane.

We next determined how Csk affects the net outcome of Lck phosphorylation of CD3ζ. Somewhat surprisingly, Csk (~500 μm⁻²) was capable of phosphorylating CD3ζ in the absence of Lck, although with a long latency (~5 min) and slow kinetics (Supplementary Fig. 7a, t½ = ~28 min). Combining Lck and Csk (both at ~500 μm⁻²) reduced the rate of CD3ζ phosphorylation by ~36%, compared to that of WT Lck alone (Supplementary Fig. 7b, t½ = 1.1 min for Lck and Csk and 0.7 min for Lck), demonstrating the inhibitory effect of Csk on Lck. At an Lck density of 50 μm⁻² (Supplementary Fig. 7c), we found a much stronger inhibitory effect of Csk on Lck (69% slower CD3ζ phosphorylation). This result is consistent with the idea that rapid Y394 phosphorylation at high Lck densities (Fig. 2) partially overrides the inhibitory effect of Csk. Consistent with a protective role of Lck pY394, the Y394F mutation led to much greater inhibitory effect of Csk, reducing the rate of CD3ζ phosphorylation by 84% (Supplementary Fig. 7b; as expected, Y505F completely abolished the effect of Csk at all Lck densities (Supplementary Fig. 7b,c)). We conclude that Csk inhibits the catalytic activity of Lck mainly through increasing Y505 phosphorylation.

We next determined how Csk alters the phase behavior for phosphorylation state of CD3ζ by using fixed concentrations of Csk (~150 μm⁻²) and CD3ζ (~400 μm⁻²) and varying the concentrations of Lck and CD45. The ‘phosphorylation-positive’ area was smaller when Csk was present (Fig. 6c), thus demonstrating the negative role of Csk. We note that whereas Csk reduced the phosphorylation signal under most conditions, it enhanced the CD3ζ phosphorylation at low densities of Lck and CD45. This apparent ‘positive role’ of Csk is because Csk alone can directly phosphorylate CD3ζ, albeit at a much slower rate than Lck (Supplementary Fig. 7a).

Membrane detachment of Csk triggers CD3ζ phosphorylation

TCR stimulation by antibody causes transient dissociation Csk from its transmembrane adaptor protein PAG (CBP)34,35. This dissociation event has been proposed to trigger or augment TCR signaling by releasing Lck inhibition34, which is consistent with recent data showing that acute inhibition of membrane-targeted Csk leads to T-cell activation16. To test this idea in our reconstitution system, we induced Csk dissociation in a controlled manner by using TVMV protease to cleave Csk from the His10-tag that anchors it to the membrane...
Protein clustering triggers CD3ζ phosphorylation

Upon TCR engagement, signaling proteins, such as Lck and ZAP-70, rapidly segregate into discrete microdomains that exclude the phosphatase CD45 (refs. 37–39), resulting in local concentration changes of these proteins. Our phase diagrams (Fig. 5c) predict that a small perturbation in the kinase or phosphatase concentration could drive a phase transition of the network, leading to net phosphorylation. To test this idea, we established an inducible expression system on the liposome surface by fusing Lck and CD45 (Fig. 6c), thereby creating multivalent heteromers of these proteins. Our phase diagrams (Fig. 7b) show that small changes in the concentration of Lck and CD45 could drive a phase transition of the network, leading to net changes of these proteins. Our work provides a complementary approach to those cellular studies by using purified enzymes in well-defined in vitro reactions. Although it lacks cellular complexity, our system provides information that is relevant for understanding how these enzymes function in living cells. First, unlike all prior in vitro studies of SFKs, our reconstitution system studies how these enzymes work on membrane surfaces, where reaction rates and, potentially, substrate presentation differ from solution measurements. Second, we used an

DISCUSSION

Here we have analyzed the TCR proximal signaling network reconstructed onto lipid bilayers, first decoupling this complex network into subreactions involving either the kinase or phosphatase and then combining components to reconstruct a more complete system. Prior studies on the mechanism of Lck, Csk and CD45 were mainly on cellular systems, using knockdown or mutation, or single-time-point phosphorylation readouts from immunoprecipitation reactions. Although such work can provide a cellular context for the roles of the enzymes, it is difficult to control enzyme states (for example, regulatory phosphorylation states of Lck) and levels and hence to obtain precise kinetic information about rates and equilibrium measurements. Our work provides a complementary approach to those cellular studies using purified enzymes in well-defined in vitro reactions. Although it lacks cellular complexity, our system provides information that is relevant for understanding how these enzymes function in living cells. First, unlike all prior in vitro studies of SFKs, our reconstitution system studies how these enzymes work on membrane surfaces, where reaction rates and, potentially, substrate presentation differ from solution measurements.
intact, native polypeptide (CD3ζ of the TCR) as a substrate for the reaction. Although this is a simplification of the multisubunit TCR complex, CD3ζ is sufficient to initiate signaling in chimeric antigen receptors and represents a more physiological substrate than denatured enolase or peptides, which have been widely used for studying SFKs. Third, when physiological concentrations of Lck, CD45 and CD3ζ in mature T cells are used in our liposome-based reaction, the network is in a quiescent state but is poised close to a threshold point for net CD3ζ phosphorylation, thus mimicking the physiological situation for this enzyme network. Importantly, by varying the concentrations of purified enzymes, combining them in different ways, and performing kinetic and steady-state readouts, we have gained new insights into the substrate specificity, enzyme regulation and how the proximal TCR signaling system behaves as a network.

**Kinase and phosphatase specificity and regulation**

The general consensus is that the kinase activity of all SFKs is differentially regulated by phosphorylation of two conserved tyrosines. This notion is based largely on end-point kinase assays of WT and tyrosine mutants of Src immunoprecipitated from cell lysates, but caused an 80% reduction in viral activity of immunoprecipitated c-Src, Y416F did not produce any effect on c-Src activity but caused an 80% reduction in viral activity. Furthermore, the tyrosine mutants of Src immunoprecipitated from cell lysates consistently found to increase the kinase activity upon phosphorylation of Y394 and of Y505, respectively, revealing a ten-fold dynamic range in Lck activity (Fig. 8). Our measurements (Fig. 3a,b) suggest an imbalance of the two opposing regulatory effects, as phosphorylation of Y394 causes only a modest activation, whereas phosphorylation of Y505 produces a much stronger repression. Perhaps more important for signaling, our results indicate that Lck activity depends on the temporal order for the two phosphorylation events. If Y505 phosphorylation occurs first, the kinase becomes essentially inactive, which greatly reduces the likelihood for subsequent Y394 phosphorylation. However, if Y394 phosphorylation occurs first, Lck remains active when subsequently phosphorylated on Y505, in agreement with a previous study on other SFKs. Thus, Y394...
phosphorylation may be more important for protecting the kinase from inactivation than for increasing kinase activity per se (<2-fold effect). This would explain a previous finding that Y394 phosphorylation is absolutely required for initiation of T-cell signaling52. Our results also reveal that conditions that favor Y505 phosphorylation over Y394 phosphorylation will lead to Lck quiescence, particularly the combination of Csk and lower Lck concentrations. (Fig. 6b and Supplementary Fig. 6b).

We also observed features of Lck regulation that were not previously anticipated. It was thought that Lck autophosphorylates Y394 in trans and that Y505 phosphorylation relies solely on the inhibitory kinase Csk23. However, we find that Lck also autophosphorylates Y505 in trans (Fig. 2). Although this result is surprising for current thinking about Lck regulation, it is consistent with previous findings that c-Src autophosphorylates Y527 (refs. 24,46). Although our and prior studies showing autophosphorylation of the inhibitory tyrosine of SFKs were performed with purified proteins, evidence suggests that this reaction may also occur in cells. In support of this idea, we have found that treatment of Jurkat T cells with a Lck-specific inhibitor (320-fold selectivity over Csk)53 leads to a reduction of up to 50% in Y505 phosphorylation (Supplementary Fig. 8). Conversely, selective inhibition of Csk in T cells does not completely eliminate Lck Y505 phosphorylation24, and 20–50% of c-Src Y527 phosphorylation persists in Csk-deficient cells3. Interestingly, unlike trans-phosphorylation of Y394, the trans-autophosphorylation of Y505 (Fig. 2c) is only modestly enhanced by confining Lck to a 2D membrane surface (Fig. 2a), perhaps owing to a sub-optimal geometry for catalysis.

Our study also has uncovered new information on the substrate specificity of CD45. We show that CD45 dephosphorylates CD3ζ >20-fold faster than Lck, suggesting that CD45’s dominant effect is reversing Lck’s phosphorylation of the TCR. However, full inhibition of CD3ζ phosphorylation requires at least ten-fold more CD45 than Lck (Fig. 5d), which may explain the very high expression of CD45 in mature T cells55. This high demand for CD45 may be due, in part, to ZAP-70 binding protecting phospho-ITAMs against CD45 dephosphorylation (Fig. 4a,c and Fig. 5g). Regarding Lck, we show that CD45 dephosphorylates Y394 and Y505 at similar rates (Fig. 4b,c). The integrated action of all three CD45-catalyzed dephosphorylation events can be visualized from the phase diagrams, which reveal that CD45 is primarily an inhibitor for TCR phosphorylation (Fig. 5c).

TCR triggering

Some elements in the T-cell signaling pathway are believed to be highly cooperative such that the transition from a quiescent to an activated T cell occurs in a switch-like manner. Our data reported here have revealed that cooperativity is at least partially encoded in the TCR proximal signaling architecture itself. A recent mathematical model12 suggested that the TCR proximal signaling ultrasensitivity arises from the multiple phospho-acceptor sites on CD3ζ, sequential phosphorylation of these sites by Lck27 and protection from dephosphorylation by ZAP-70. We have obtained experimental support for ZAP-70’s protection against CD45 dephosphorylation (Fig. 4a,c) but found that the ability of ZAP-70 to bind phospho-ITAMs did not alter the cooperativity of the kinase-phosphatase reaction (Fig. 5g). We speculate that at least part of the switch-like behavior observed in this study may be due to the distributive multisite phosphorylation of CD3ζ by Lck. Distributive phosphorylation has been suggested to produce ultrasensitivities in other kinase cascades56, especially when both the substrate and enzyme are confined to the membrane57.

In this framework, increasing the number of phosphorylation sites (for example, using an intact TCR complex that contains ten ITAMs) might enhance the degree of ultrasensitivity.

Additional mechanisms for achieving greater ultrasensitivity could include spatial exclusion of CD45 from the tight contact zone of T lymphocytes and APCs38,39, as proposed in the ‘kinetic segregation model‘38,39. Consistent with this idea, phosphorylation of CD3ζ was more sensitive to variation in CD45 concentration (Fig. 5f) than in Lck concentration (Fig. 5e). We also show that inducing the clustering of Lck and CD3ζ using a chemically inducible FKBP-FRB aggregation system triggered a dramatic, global phase transition of strong TCR phosphorylation at physiological levels of Lck and CD45 (Fig. 7b).

In the future, other elements in the T-cell signaling pathway, such as the adaptor protein LAT and its associated factors, could be added to reconstituted liposomes to see how they affect the threshold and sensitivity of the TCR or downstream phosphorylation reactions.

METHODS

Methods and any associated 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.

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AUTHOR CONTRIBUTIONS

E.H. and R.D.V. designed the study. E.H. collected the data and conducted the analyses. E.H. and R.D.V. wrote the manuscript.

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ONLINE METHODS

Materials. Synthetic 1,2-dioleoyl-sn-glycer-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycer-3-phospho-L-serine (POPS), 1,2-dioleoyl-sn-glycer-3-{N-[5-(s-acetyloxyphenyl)iminodidicarbonyl]acetyl}(nickel salt, DGS-NTA-Ni) and N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl-sn-glycer-3-phosphoethanolamine (Rhod-PE) were purchased from Avanti Polar Lipids. SNAP-cell S05 was obtained from New England Biolabs. Tetramethylrhodamine-5-maleimide was from Life Technologies. Lck inhibitor (7-cyclopentyl-5-(4-phenoxypyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine) and rapamycin were obtained from Sigma-Aldrich. The antibodies used in this study were: mouse anti-pY142-CD3ζ (anti-CD247, 558489, 1:1,000, BD PhosFlow), anti-pY416-Src (pY394-Lck, 56094, BD PhosFlow 1:500), anti-pY505-Lck (558552, BD PhosFlow, 1:3,000), anti-GAPDH (MAB374, Millipore, 1:50,000), rabbit anti-Lck (2984S, Cell Signaling, 1:1,000) and rabbit anti-pY416-Src (2101S, Cell Signaling, 1:1,000). Validation of each antibody is provided on the manufacturer website.

Recombinant protein expression and purification. cDNA encoding the cytosolic domain of human CD3ζ (amino acids aa 52–164) was subcloned into a pET28a vector. The coding sequence of the His6 tag in pET28a was modified to His10 via QuikChange site-directed mutagenesis with KOD polymerase (Millipore). A Lys-Cys-Lys-Lys sequence was inserted between His10 and CD3ζ for fluorescent labeling of the protein product. Full-length (FL) human Lck, FL human Csk and the cytosolic portion of human CD45 (aa 598–1304) were subcloned into a modified pFastBacHTA vector containing His10 upstream to the multiple cloning site. For Lck, a C2A point mutation was introduced to prevent the N-terminal myristoylation and tyrosine mutants were generated by QuikChange. To create Lck or CD3ζ fusion proteins containing FKBP or FRB repeats, FKBP-L or FRB-L was flanked by XbaI and SpeI site, and repetitively cloned into the vector backbone at the SpeI site. ‘L’ denotes a 12-aa flexible linker (GSGSGGGGSGSS). The SNAP-ISH2 reporter construct was generated by fusing the SNAP-tag (New England BioLabs), a 14-aa flexible linker (GSGSGGGGSGSSSTR) and the tandem SH2 domains of human ZAP-70 (aa 1–259), and cloned into pGEX6P-2 vector via BamHI and EcoRI. Sequences of the primers for cloning are available upon request.

His10-KckCD3ζ were bacterially expressed in BL21 (DE3) strain of Escherichia coli. His10-tagged Lck, CD45, Csk and all their fusion proteins were expressed in SF9 cells using the Bac-to-Bac baculovirus system (Life Technologies). The cells were lysed in an Avestin Emulsiflex system. All His10 proteins were purified by using Ni-NTA agarose essentially as described60. SNAP-ISH2 was expressed in BL21 (DE3) as a glutathione S-transferase (GST) fusion protein and purified by using glutathione-Sepharose beads (GE Healthcare) as described61. Soluble SNAP-ISH2 was generated by cleaving the GST moiety via the PreScission Protease.

For dephosphorylation of recombinant Lck as shown in Supplementary Figure 2d, the cytosolic portion of CD45 (aa 598–1304) was fused with a GST tag and PreScission recognition site at its N terminus and cloned into a modified pFastBacHTA vector lacking the coding sequence of the polyhistidine tag. The GST fusion protein (GST-CD45) was expressed in SF9 cells and purified using glutathione-Sepharose beads (GE Healthcare) as described62. Soluble SNAP-ISH2 was generated by cleaving the GST moiety via the PreScission Protease.

Protein labeling. To prepare the fluorescent reporter for FRET assays, 10 µM SNAP-ISH2 was incubated with an equal concentration of SNAP30 in HBS buffer for 1 h at room temperature or overnight on ice in the presence of 1 mM TCEP. For solution-based FRET (Fig. 1d,e), 10 µM His10-KckCD3ζ was incubated with 30 µM tetramethylrhodamine-5-maleimide in HBS buffer for 1 h at room temperature. The reaction was quenched by 1 mM DTT and the excess dyes were removed by extensive dialysis. The labeling efficiency was 80–90%.

Liposome preparation. Phospholipids (79.7% POPC + 10% POPS + 10% DGS-NTA-Ni + 0.3% Rhod-PE) were dried under a stream of N2, desiccated for 3 h and suspended in 1× kinase buffer. Large unilamellar liposomes were prepared by extrusion through a pair of polycarbonate filters with a pore size of 200 nm, according to the manufacturer instructions (Avanti Polar Lipids). For liposomes used in Supplementary Figure 1c, the changes in PS content were compensated by opposite changes in PC content.

Pre-phosphorylation and dephosphorylation of Lck and CD3ζ. Recombinant Lck from SF9 cells was partially phosphorylated on Y505 (Fig. 2a, time 0). To prepare a homogeneous population of unphosphorylated Lck, freshly purified Lck was incubated with GST-CD45 on ice for at least 12 h for complete dephosphorylation (Supplementary Fig. 2d). MS revealed 0% phosphorylation on Y394 and 1.5% phosphorylation of Y505 after this treatment (E.H. and R.D.V., unpublished observations). The CD45 phosphatase was pulled down by glutathione-Sepharose beads, and the supernatant containing Lck and residual GST-CD45 was subjected to gel filtration for further separation. To pre-phosphorylate Lck on one or both regulatory tyrosines (Y394 and Y505), freshly purified Lck (WT, Lck (Y394F) or Lck (Y505F)) was concentrated using Amicon Ultra Centrifugal Filter units (Millipore) to >5 µM, and mixed with 1 mM ATP in 1× kinase buffer (50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 1 mM TCEP) on ice for >24 h to allow complete autophosphorylation, as confirmed by WB (Supplementary Fig. 2e,f). ATP was subsequently removed by gel filtration, and monomeric protein fractions were pooled, snap frozen in liquid N2 and stored at −80 °C. In the case of WT Lck, MS analysis revealed 82% phosphorylation for Y394, and 97% phosphorylation for Y505 (Supplementary Fig. 3). Notably, the immunoblot analyses have revealed that net phosphate loss of Lck on both regulatory tyrosines can occur at relatively low ATP concentrations (Supplementary Fig. 2). Therefore, it is important to incubate Lck with excess concentrations of ATP to achieve maximum and stable level of phosphorylation.

To pre-phosphorylate CD3ζ for studying the substrate specificity of CD45, His10-KckCD3ζ was mixed with His10-Lck and ATP in 1× kinase buffer on ice. Aliquots of reactions were terminated at different time points, and CD3ζ phosphorylation was considered complete when no further changes in electrophoretic mobility were observed. Phosphorylated CD3ζ was then separated with Lck by gel filtration.

Reconstitution assay of membrane-bound recombinant proteins. Indicated concentrations of His10-tagged proteins were premixed and then incubated with DGS-NTA-Ni containing liposomes for 40–60 min to allow for membrane attachment. Following 40 min incubation, His10 proteins were undetectable in the supernatant after liposome sedimentation, establishing complete membrane attachment.

FRET assays were carried out at room temperature in solid white, 96-well polystyrene plates (Corning) in a total volume of 100 µl per reaction. All protein components (including 0.3 mg/ml BSA to prevent nonspecific binding) were mixed, then liposomes were added and the mixture was incubated for 40–60 min, during which the SNAP30 fluorescence was monitored at the minimal intervals (6–30 s, depending on the number of wells monitored) with 504-nm excitation and 540-nm emission. During this period, His10 proteins bind to the liposomes and the temperature equilibrates, establishing a stable baseline. ATP was subsequently injected followed by 5 s of automatic shaking of the plate, and the fluorescence was further monitored for at least 1 h. Data were normalized by setting the average fluorescence value of the last 10 data points before ATP addition as 100% and background fluorescence as 0%.

Immunoblot analyses of phosphorylation and dephosphorylation. The time course of both phosphorylation (Figs. 2a–c and 6a,b, Supplementary Figs. 2 and 6) and dephosphorylation (Fig. 4a) of Lck was followed by immunoblotting, using mouse monoclonal antibodies (mAbs) against pY394 or pY505 of Lck. Autophosphorylation was triggered by 1 mM ATP unless indicated otherwise. For experiments shown in Figure 4c, Lck was reconstituted onto the liposomes at 500 molecules per µm2, and autophosphorylation was triggered by indicated concentrations of ATP, terminated after 5 min incubation at room temperature and analyzed by WB. The phosphorylation level at 5 min was used as a proxy for the initial rate of autophosphorylation to estimate the ATP Ks. Experiments shown in Figure 4a were conducted in solution at room temperature, and dephosphorylation was initiated by adding CD45 (0.1 µM) to a mixture containing equal concentration.
(0.1 μM) of both pre-phosphorylated CD3ζ (pCD3ζ) and pre-phosphorylated Lck (pLck). In all kinetic measurements using immunoblotting, aliquots of reactions were removed and terminated by treating with 1× SDS sample buffer at indicated times and boiled for 5 min before analysis by SDS-PAGE and WB using standard protocols. Blots were quantified by ImageJ (http://rsb.info.nih.gov/ij/).

Electrophoretic mobility assay for CD3ζ phosphorylation. For experiments shown in Figure 3c,d, DGS-NTA-Ni containing liposomes (1 mM total lipids) were incubated with 20 nM His10-Lck plus 2.54 μM or 7.62 μM His10-Lck (pCD3ζ) for 1 h at room temperature to achieve the following protein densities: −58 μm² for His10-Lck; −7,300 μm² or −22,000 μm² for His10-CD3ζ. After the addition of 1 mM ATP, the reaction was terminated at indicated time points and subjected to SDS-PAGE followed by Coomassie staining.

**Rapamycin-induced protein clustering on membranes.** For rapamycin-induced CD3ζ-Lck co-clustering (Fig. 7a,b) the two proteins were fused with repeats of FKBP and FRB, respectively, designated as (FKBP)₆-CD3ζ and (FRB)₄-Lck. These fusion proteins were purified with an N-terminal His10-tag and co-attached to liposomes with CD45. Rapamycin was added 5 min before the addition of ATP to induce CD3ζ-Lck co-clustering. To measure phase diagrams, the density of (FRB)₄-Lck and CD45 was varied, and the density of (FKBP)₆-CD3ζ was fixed, similarly to the experiments shown in Figure 5b. To achieve rapamycin-induced clustering of CD3ζ alone as shown in Figure 7c, 160 μm² (FKBP)₆-CD3ζ and 240 μm² (FRB)₄-CD3ζ was co-attached to liposomes with various densities of Lck and CD45 (as described in Fig. 5b), followed by sequential addition of rapamycin and ATP. The phase-diagram data were measured at 1 h after ATP addition. A 2:3 molar ratio of (FKBP)₆-CD3ζ and (FRB)₄-CD3ζ was used to equal moles of FKBP and FRB. Final rapamycin concentration was approximately three-fold the bulk FKBP concentration. We noted that the efficiency for rapamycin induced clustering exhibits a bell-shaped dependence to rapamycin concentration (E.H. and R.D.V., unpublished observation).

**Liquid chromatography mass spectrometry.** Protein samples were acetone precipitated, followed by reconstitution in freshly prepared 8 M urea, 0.2% protease max (Promega), pH 8.0. To mitigate the instability of the phosphatase group, as suggested by Supplementary Figure 2e, samples were not reduced or alkylated. The urea concentration was diluted to <1 M by the addition of 50 mM ammonium bicarbonate, followed by the addition of trypsin at a 1:25 ratio, and allowed to digest for 30 min at 50°C. A portion of the digest was removed and the reaction was quenched by lowering the pH to <3 using 10% formic acid. The samples were cleaned using C₁₈ microspin columns (NEST group) and peptides were eluted in 60% acetonitrile, 39.9% water and 0.1% formic acid. The samples were dried briefly in a speed vacuum and reconstituted in liquid chromatography (LC) mobile phase A.

The LC system was a Proxeon (Thermo) Easy nLC II, run at 0.3 μl per minute, where the column was a self-packed C₁₈ 3-μm matrix packed to 15-cm length and the inner diameter was 100 μm. The mass spectrometer was an LTQ Orbitrap Velos in which data was acquired in a data-dependent fashion, with the top 12 most intense multiply charged precursor ions selected for fragmentation in the linear ion trap. Data were searched against a custom database containing the sequence of human Lck. Data analysis and extraction, including MS/MS TIC and feature extraction, were performed using Scaffold (Proteome Software v. 4.05).

**Cell culture and Lck inhibitor treatment.** Jurkat T-cell line was kindly provided by A. Weiss (University of California, San Francisco). Cells were grown in RPMI-1640 (Sigma-Aldrich) supplemented with 5% FBS (Life Technologies), 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37°C with 5% CO₂. At a density of 3 × 10⁷ cells per milliliter, Jurkat cells were treated with 100 nM cell-permeable Lck-specific inhibitor ([4-aminoo-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-7-y1-cyclopenatone, Sigma-Aldrich]) at 37°C for 0.5 h, 6 × 10⁷ cells were then harvested by centrifugation at 600 × g for 3 min and resuspended in 60 μl lysis buffer (50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.2% SDS, 1 mM Na₃VO₄, 10 mM NaF, 1 mM phenylmethanesulfonylfluoride and 1× complete protease inhibitor (Roche)). After 15 min incubation on ice, cell lysates were centrifuged at 16,000 × g for 10 min; supernatants were then removed and mixed with SDS sample buffer, boiled for 5 min and subjected to SDS-PAGE and WB.

**Protein-density calculation.** To calculate the surface density of proteins in cells, we assume T cells adopt a spherical shape with 7 μm diameter and that all proteins are present on the plasma membranes with a roughness factor of 1.8 (ref. 62). Hence, 10,000 molecules per cell corresponds to ~36 molecules per μm² surface density. The expression levels for Lck, CD45 and TCR have been previously determined and often reported as number of molecules per cell. The number of Lck varies from 40,000 to 120,000 in T cells63. CD45 expression levels vary from 100,000 to 500,000 molecules per cell; TCR level was measured to be 30,000–100,000 molecules per cell64–66. On the basis of these previous measurements and the assumptions above, we estimated the physiological densities for the relevant proteins as follows: CD45, 361–1,805 μm⁻²; Lck, 144–433 μm⁻²; TCR, 108–361 μm⁻².

One T cell normally expresses ~50,000 ZAP-70, on the basis of quantitative immunofluorescence measurement (A. Weiss, personal communication). To mimic physiological conditions, we maintained a 1:1 SNAP505-tSH2/CD3ζ molar ratio in our phase diagram measurements as shown in Figures 5–7 and Supplementary Figure 5.

In our reconstitution system, the surface density of a given protein was calculated by dividing the number of protein molecules by the total area of the exposed membrane (outer leaflet) of liposomes. Assuming a 5-nm thickness of the phospholipid bilayer66, the inner diameter of a 200-nm liposome is 190 nm; therefore, ~52.6% of total lipids are present in the outer membranes of liposomes (i.e., for 1 μmol lipids, ~0.526 μmol is exposed to proteins). Assuming that each phospholipid occupies an area of 0.65 nm² (ref. 67), the exposed membrane area should be ~2.06 × 10⁴ μm² per micromole of total lipids. On the basis of the area of occupancy for each phospholipid molecule (0.65 nm²), we can also calculate the total number of lipids per 200-nm liposome (367,000), thereby allowing the calculation of liposome concentration from total lipid concentration (Supplementary Fig. 1b).

**Data analyses.** The heat maps in Figures 5–7 were created in MATLAB 2012b. The percentage of fluorescence quenching at 1 h after ATP (Fig. 5b) was used to create a 3D scatter plot with CD45 and Lck densities as the x and y axes, respectively. The scatter plot was then subjected to Delaunay triangulation (tri = delaunay (x,y)), after which a 3D surface plot was generated using the built-in function ‘trisurf’. Finally, the 3D surface plot was subjected to rotation to generate the heat map. All dose-response data were analyzed with GraphPad Prism 5.0.

**Original images of immunoblots.** Original images of immunoblots used in this study can be found in Supplementary Figure 9.