TRANSMEMBRANE ADAPTOR PROTEINS: ORGANIZERS OF IMMUNORECEPTOR SIGNALLING

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Signalling through multichain immunoreceptors is required for the development, activation and differentiation of haematopoietic cells. One of the most challenging questions with regard to these processes is how immunoreceptor-mediated signals are converted into appropriate cellular responses. The recent identification of a novel group of signalling molecules, known as transmembrane adaptor proteins, has led to exciting new insights. The transmembrane adaptor proteins LAT, NTAL, PAG, LIME, TRIM, SIT and LAX organize complex membrane-proximal signalling assemblies and are therefore key mediators of immunoreceptor-mediated signalling. As we discuss here, their identification has greatly facilitated our understanding of how immunoreceptors are connected to intracellular signalling cascades.

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doi:10.1038/nri1414

Many well-orchestrated signals regulate the development, activation and differentiation of haematopoietic cells. Among the molecules that are involved in these processes, a group of signal-transducing receptors, known as multichain immunoreceptors, have a key role. This group comprises the T- and B-cell receptors (TCR and BCR), most Fc receptors and the collagen receptor expressed by platelets (BOX 1). Multichain immunoreceptors have similar structural and signalling principles in that they consist of a ligand-recognition module and non-covalently associated signalling subunits. The hallmark of the cytoplasmic domain of the signalling subunits is the presence of at least one IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM)[1,2].

Following the interaction of the recognition unit with its cognate ligand, the connection between the engaged receptor and the intracellular environment is initiated by phosphorylation of two core tyrosine residues within the ITAM(s) of the signal-transducing subunit(s). This is achieved by protein tyrosine kinases (PTKs) of the SRC-KINASE FAMILY, such as LCK and FYN in T cells and natural killer (NK) cells, or LYN and FYN in B cells and mast cells[3].

Originally, it was assumed that SRC kinases are associated, albeit with low stoichiometry, with the cytoplasmic tails of the signalling chains. Ligand-induced aggregation of the receptor complexes was thought to bring these kinases together, which then resulted in their transphosphorylation of one another and, in this way, initiation of the signalling process. Therefore, immunoreceptors were thought to function similarly to receptors with intrinsic PTK activity, such as the insulin receptor or platelet-derived growth factor receptor.

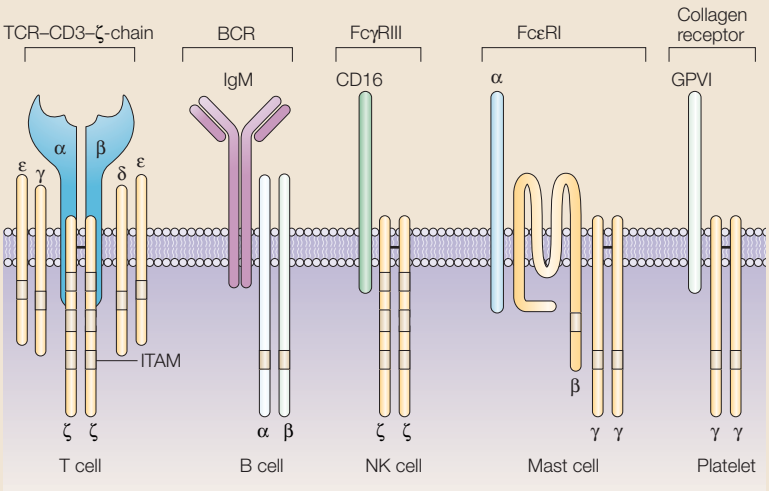
Although this model has not been disproved, a new and not necessarily mutually exclusive model has emerged that seems to provide a better explanation for some features of the early phases of immunoreceptor signalling. According to this model[4,5], aggregates of immunoreceptors, which are formed after interaction with ligands, move to lipid rafts (also known as glycosphingolipid-enriched microdomains, GEMs) (BOX 2), which contain SRC kinases. Subsequently, the ITAMs of the signalling subunits become phosphorylated and then function as binding sites for the SRC HOMOLOGY 2 (SH2) DOMAINS of SYK-FAMILY KINASES (spleen tyrosine kinase family), such as ZAP70 (ζ-chain-associated protein kinase of 70 kDa) in T cells (FIG. 1). The SYK-family PTKs are

IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM). A structural motif containing tyrosine residues that is found in the cytoplasmic tails of several signalling molecules. The motif has the form $YXX(L/I/V)X_{6-8}YXX(L/I/V)$, where X denotes any amino acid. The tyrosine residues are targets for phosphorylation by SRC-family protein tyrosine kinases and subsequent binding of proteins that contain SRC homology 2 (SH2) domains.

thereby also recruited to rafts, where they become phosphorylated and activated by SRC kinases. Importantly, SRC kinases are not the only signalling molecules to reside constitutively in rafts. Several other components of the earliest phases of immunoreceptor signalling are also present constitutively: for example, the LCK-associated T-cell co-receptors CD4 and CD8, and the membrane phospholipid phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$).

An important question asked by immunologists concerns how the initial immunoreceptor-mediated signal is further propagated. It was well known that several cytoplasmic signalling proteins with SH2 domains translocate from the cytosol to the plasma membrane immediately after stimulation through the immunoreceptor. This indicated the existence of membrane-associated molecules that are capable of binding different SH2 domains after tyrosine phosphorylation. The search for proteins that have these properties led to the identification of a highly specialized group of integral membrane proteins, the main task of which is to provide multiple docking sites for SH2-domain-containing cytosolic signalling and effector molecules. These proteins are known as transmembrane adaptor proteins (TRAPs). Their identification has greatly facilitated our understanding of the molecular organization of membrane-proximal signalling complexes in haematopoietic cells. Here, we summarize the current knowledge of this novel group of signalling molecules.

Box 1 | **Molecular composition of haematopoietic immunoreceptors**



The figure shows the following immunoreceptors: the T-cell receptor (TCR), which uses the CD3–ζ-chain complex for signalling; the B-cell receptor (BCR), which interacts with the signal-transducing subunits Igα (also known as CD79a) and Igβ (also known as CD79b); the low-affinity Fc receptor for IgG (FcγRIII; also known as CD16), which is expressed by natural killer (NK) cells and associates with dimers of either the ζ-chain or the structurally closely related γ-chain (the IgE Fc receptor γ-subunit); the high-affinity Fc receptor for IgE (FcεRI), which mediates mast-cell degranulation after the binding of IgE and uses a β-chain and the γ-chain for signal transduction; and the collagen receptor, which is expressed by platelets and consists of the ligand-binding subunit glycoprotein VI (GPVI) and the signal-transducing γ-chain (the same γ-chain that is used by FcγRIII and FcεRI). Other immunoreceptors that are structurally similar to FcγRIII or the collagen receptor include the following: the high-affinity receptor for IgG (FcγRI; also known as CD64) and the high-affinity receptor for IgA (FcαR; also known as CD89), which are expressed by monocytes and macrophages; and the more distantly related NK-cell- and myeloid-cell-activating receptors, such as members of the killer-cell immunoglobulin-like receptor (KIR), signal-regulatory protein (SIRP), CD200, NK-cell protein (NKP), myeloid-associated immunoglobulin-like receptor (MAIR), NK group 2 (NKG2) and lymphocyte antigen 49 (LY49) families, which associate with either the DAP12 (DNAX activation protein 12) or DAP10 signal-transducing chains[89,90]. As shown, all immunoreceptor-associated signalling components have immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains. ITAMs have the consensus sequence $YXX(L/I/V)X_{6-8}YXX(L/I/V)$ (where X denotes any amino acid) and are present in varying numbers in the imunoreceptor-associated signalling subunits[1]. It should be noted that the ζ- and γ-chains have only short extracellular domains that probably do not recognize any ligands, whereas other signalling subunits (such as CD3γ, CD3δ, CD3ε, Igα and Igβ) are more receptor-like and have longer extracellular domains of mostly unknown function.

TRAPs: structure, general function and history

The structure of TRAPs is strikingly similar to the immunoreceptor-associated ζ-chain and γ-chain (FIG. 2). All TRAPs identified so far have only short extracellular domains and relatively long cytoplasmic tails that contain a variable number of tyrosine residues within tyrosine-based signalling motifs. However, none of the known TRAPs have classical ITAMs, and in general, TRAPs do not associate directly with immunoreceptors; an exception might be the TCR-interacting molecule (TRIM) (discussed later).

Nevertheless, the general function of TRAPs is similar to the ζ- and γ-chains. After ligation of immunoreceptors and other signal-transducing receptors, tyrosine residues that are present in the cytoplasmic domains of TRAPs become phosphorylated and then bind SH2-domain-containing cytoplasmic signalling or effector proteins. These features allow the TRAPs to organize signalling complexes close to the plasma membrane and therefore to connect signal-transducing receptors with intracellular signalling pathways, which then allows the induction of appropriate cellular responses. The identification of TRAPs has therefore answered the question of how key cytosolic signalling proteins become recruited to the plasma membrane after the ligation of immunoreceptors.

The history of TRAPs is brief: the first TRAPs, LAT (linker for activation of T cells[6,7]) and TRIM[8], were described in 1998, and since then, five others have been identified. Four of the known TRAPs reside in lipid rafts: PAG (protein associated with GEMs; also known as carboxy (C)-terminal SRC kinase (CSK)-binding protein, CBP), LAT, NTAL (non-T-cell activation linker; also known as linker for activation of B cells, LAB) and LIME (LCK-interacting membrane protein). By contrast, the other three TRAPs are mainly non-raft proteins: TRIM, SIT (SH2-domain-containing protein tyrosine phosphatase (SHP2)-interacting TRAP) and LAX (linker for activation of X cells, where X denotes an as yet unidentified cell).

LAT

LAT was not only the first TRAP to be described but also seems to be by far the most important. Indeed, LAT can be considered the master switch of T-cell activation. The

SRC-KINASE FAMILY
A group of structurally related cytoplasmic and/or membrane-associated enzymes named after its prototypical member, SRC. In haematopoietic cells, SRC kinases — such as LCK, FYN and LYN — are the first protein tyrosine kinases to become activated after stimulation through the immunoreceptor. Among other substrates, they phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) that are present in the signal-transducing subunits of the immunoreceptors, thereby providing binding sites for SRC homology 2 (SH2)-domain-containing molecules, such as spleen tyrosine kinase (SYK).

SRC HOMOLOGY 2 (SH2) DOMAINS
Protein domains that are found commonly in signal-transduction molecules. They specifically recognize phosphotyrosine-containing peptide sequences in proteins.

essential role of LAT in T-cell signalling was first indicated by analysing LAT-deficient variants of the Jurkat T-cell line[9,10]. In these cells, it was found that key elements of the TCR-mediated signalling cascades that lead to interleukin-2 (*IL-2*)-gene expression are completely abolished, including flux of calcium ions, activation of RAS–extracellular-signal-regulated kinase (ERK), expression of CD69 and activation of the transcription factor nuclear factor of activated T cells (NFAT). All of these defects can be reconstituted by re-expression of wild-type LAT. By contrast, LAT variants with mutations in the juxtamembrane CXXC palmitoylation motif (where X denotes any amino acid), which is responsible for lipid-raft targeting of LAT, fail to rescue signalling[11]. Similarly, treatment of T cells with polyunsaturated fatty acids leads to the displacement of LAT from lipid rafts and profoundly inhibits signalling through the TCR[12]. These data indicate that the association of LAT with rafts is essential for its proper function.

LAT is not only important for the activation of peripheral T cells but is also essential for T-cell maturation in the thymus. Disruption of the *Lat* gene results in an early block in thymocyte development (at double-negative stage 3, DN3); therefore, most thymic T cells do not reach the double-positive stage, at which selection processes occur in normal animals[13]. This results from a loss of signalling capacity of the pre-TCR, which is required for early thymic development.

Box 2 | **Lipid rafts**

Most plasma-membrane lipids contain polyunsaturated fatty acids, which form a relatively uniform lipid bilayer[91]. However, the plasma membrane also contains submicroscopic regions that are enriched in sphingolipids (sphingomyelin and glycosphingolipids) and cholesterol. These regions are known as lipid rafts or glycosphingolipid-enriched microdomains (GEMs). The rafts are mainly held together by hydrophobic interactions between the long saturated fatty-acid residues of the sphingolipids. In the presence of optimal amounts of cholesterol, the sphingolipids form a specific 'ordered liquid phase', the physical properties of which are different from the rest of the plasma membrane. A hallmark of rafts is that they withstand solubilization by 'standard' detergents, such as Triton X-100. The high lipid content of the detergent-insoluble rafts allows their purification by density-gradient ultracentrifugation of detergent-solubilized cells or cell membranes[92].

Most transmembrane proteins are excluded from rafts. However, several lipid-modified molecules can be found in rafts[5], including the following: the co-receptors CD4 and CD8; the adhesion receptor CD44; members of the tumour-necrosis-factor-receptor family; and various transmembrane adaptor proteins (TRAPs), including LAT (linker for activation of T cells), NTAL (non-T-cell activation linker), LIME (LCK-interacting membrane protein) and PAG (protein associated with GEMs). In addition, several cytoplasmic molecules that have lipid modifications accumulate in rafts, such as SRC-family protein tyrosine kinases, and heterotrimeric and small G proteins. Extracellularly oriented proteins that are anchored in the membrane through glycosylphosphatidylinositol (a glycolipid moiety), such as THY1, LY6 (lymphocyte antigen 6), CD14, CD48, CD55 and CD59, also accumulate in rafts.

Because of their small size, lipid rafts cannot be directly observed by light microscopy, although some advanced techniques allow visualization of their size and shape on the cell surface[93,94]. It is possible that 'elementary rafts' are small (with a diameter of less than 10 nm) and contain few protein molecules, or perhaps only a single molecule, surrounded by a 'shell' of several hundred specific lipid molecules[95]. These units probably coalesce into larger patches after crosslinking of their protein and/or glycolipid components by antibodies or natural, multivalent ligands.

The phenoptypes of LAT-deficient Jurkat T cells and *Lat*-knockout mice reflect the ability of LAT to organize a signalling complex that couples the TCR to the main intracellular signalling pathways that regulate *IL-2*-gene transcription (FIG. 3). One pathway is based on the ordered activation of the small G protein RAS — through the connection LAT–growth-factor-receptor-bound protein 2 (GRB2)–son of sevenless homologue (SOS) — which leads to activation of the protein kinase RAF and results in activation of ERK[6]. Another LAT-controlled pathway involves the GRB2-related adaptor protein (GADS), SH2-domain-containing leukocyte protein of 76 kDa (SLP76) and IL-2-inducible T-cell kinase (ITK), which is a member of the TEC-KINASE FAMILY[14–21]. This pathway induces the activation of phospholipase C-γ1 (PLC-γ1) (REFS 22,23). PLC-γ1 then hydrolyses PtdIns(4,5)P_2, producing the second messengers inositoltrisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ initiates an increase in the cytoplasmic calcium concentration, whereas DAG is an activator of protein kinase C (PKC) isoenzymes and RAS guanyl-releasing protein (RASGRP). In addition to GRB2 and SOS, RASGRP is also an upstream activator of RAS, and there is accumulating evidence that RASGRP has a dominant role in TCR-mediated activation of ERK[24–26].

The LAT–PLC-γ1 interaction is important for immune homeostasis. This has been demonstrated in knock-in mice in which the PLC-γ1-binding site of LAT, tyrosine 136, was mutated to a phenylalanine residue (LAT^{Y136F}) (REFS 27,28). In these mice, TCR-mediated phosphorylation of PLC-γ1 is reduced, and T cells that express the mutant LAT^{Y136F} are unable to increase cytoplasmic calcium concentrations in response to signalling through the TCR. As a result, thymic development of LAT^{Y136F}-mutant mice is markedly impaired. One would expect this signalling defect to cause severe immunodeficency. However, surprisingly, TCR-mediated activation of ERK is mostly normal in these mice[27], and they have high numbers of constitutively activated $CD4^+$ T cells in the periphery. These produce T helper 2 (T_H2) cytokines and thereby promote B-cell maturation and isotype switching[27,28]. As a result, LAT^{Y136F}-mutant animals develop an autoimmune disease and have greatly enlarged peripheral lymphoid organs due to lymphocyte infiltration[27,28].

Although altered thymic development in the LAT^{Y136F}-mutant mice can be explained by the defects in activation of PLC-γ1, it is difficult to understand why the mutant T cells elicit normal ERK activation and cause autoimmunity[26,27]. Perhaps PLC-γ1 becomes weakly activated by an indirect interaction between LAT and the GADS–SLP76 complex, and consequently, it might produce a sufficient amount of DAG to activate RASGRP. Alternatively, activation of the RAS–ERK pathway might be mediated sufficiently through the LAT–GRB2–SOS pathway. Finally, it might be that ERK activation is not mediated by LAT under these conditions but instead by an unknown molecule.

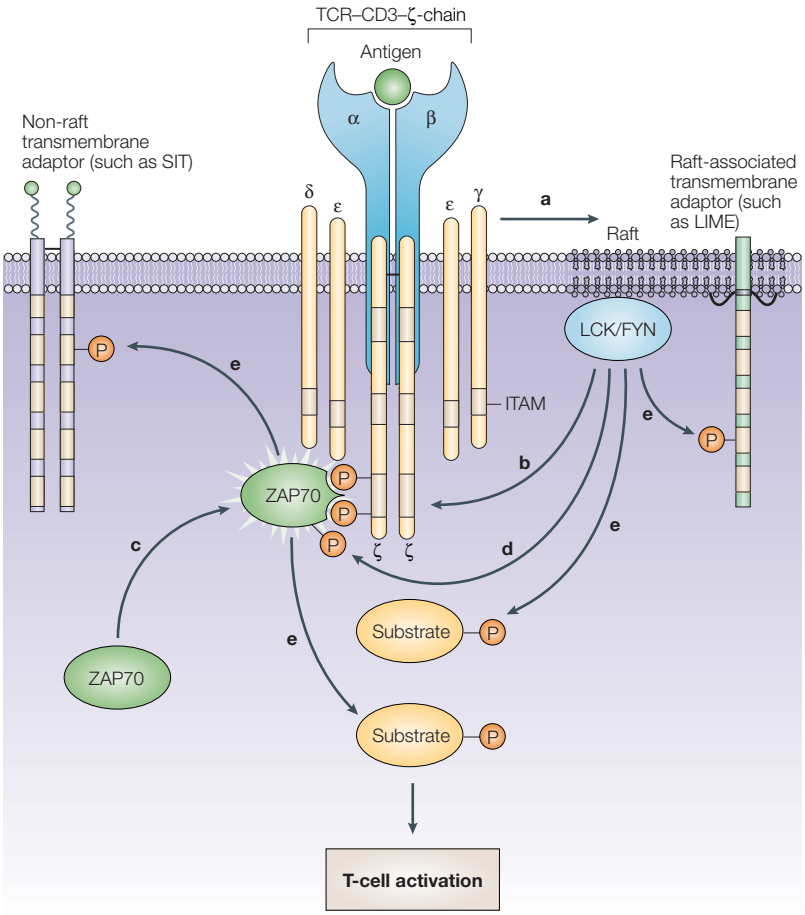


Figure 1 | **Current model of membrane-proximal immunoreceptor-mediated signalling, using the TCR as an example. a** | After binding of antigen, the T-cell receptor (TCR) moves into lipid rafts. **b** | Subsequently, protein tyrosine kinases (PTKs) of the SRC family (LCK and FYN in T cells) become activated and phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR-associated CD3–ζ-chain complex. (It should be noted that the mechanisms that lead to the activation of these enzymes are still unclear, as discussed in the text.) **c** | Phosphorylated ITAMs provide docking sites for the spleen tyrosine kinase (SYK)-family member ZAP70 (ζ-chain-associated protein kinase of 70 kDa), which binds through its two tandem SRC homology 2 (SH2) domains. **d** | When it is brought into proximity of the TCR, ZAP70 becomes phosphorylated and activated by SRC kinases. **e** | Together, activated SRC kinases and ZAP70 can then phosphorylate various cellular substrates, including transmembrane adaptor proteins, thereby connecting the TCR to the intracellular signalling machinery. LIME, LCK-interacting membrane protein; SIT, SH2-domain-containing protein tyrosine phosphatase (SHP2)-interacting transmembrane adaptor protein.

One hypothesis to explain the lymphoproliferative disease of the LAT^{Y136F}-mutant mice is that blunted TCR-mediated signalling, although it blocks thymic development, results in a failure to completely eliminate self-reactive T cells by negative selection. These non-deleted and autoreactive cells could then escape to the periphery and cause the autoimmune syndrome. However, another possibility could be that, in the periphery, LAT not only mediates positive regulatory signals for T-cell activation, but is also involved in negative signalling. Indeed, it seems that Y136 could be involved in mediating an indirect interaction between LAT and the E3 UBIQUITIN LIGASE CBL[29]. Although this has not yet been shown experimentally, LAT-bound CBL might target LAT complexes in activated T cells for proteasomal degradation. A failure to initiate this pathway could lead to dysregulation of T-cell activation and consequently to autoimmunity. Finally, impaired development and/or altered functions of CD4$^+$CD25$^+$ regulatory T (T_{Reg}) cells, as a result of altered signalling through the TCR, could underly the phenotype of the LAT^{Y136F}-mutant mice.

The complexity of LAT-mediated signalling became further evident after a recent analysis of knock-in mice that express a LAT mutant in which the three distal tyrosine residues were eliminated[30]. As expected, this mutation of LAT also almost completely abolishes thymic maturation of αβ T cells, probably because the GADS–SLP76 complex that is required for the activation of PLC-γ1 cannot be recruited. By contrast, the γδ T CELLS of these LAT-mutant mice develop normally and populate the periphery; however, surprisingly, these cells also show an activated phenotype, produce T_H2 cytokines and cause a lethal lymphoproliferative syndrome[30]. The molecular mechanisms underlying this LAT-mutant phenotype are as yet unclear, but they might involve defects in the recruitment of negative regulatory molecules to LAT. For example, it has recently been shown that phosphorylated LAT can interact through GRB2 with the adaptor protein GRB2-associated binding protein 2 (GAB2), thereby recruiting the tyrosine phosphatase SHP2, which then downregulates T-cell activation[31,32]. A failure to recruit this negative regulatory module to the membrane could result in dysregulation of T-cell activation and consequently lymphoproliferation and autoimmunity.

We still have much to learn to understand the complex biology of LAT-mediated signalling. Nevertheless, analysis of mice that express either of the two LAT mutants discussed indicates that LAT controls the development of both αβ and γδ T cells and that the signals required for αβ and γδ T-cell maturation are mediated by different regions of LAT and thereby by different molecules that associate with LAT.

LAT in non-T cells

In addition to its central role in T-cell activation and immune homeostasis, LAT might also be important in other immunoreceptor-mediated signalling pathways. For example, tyrosine residues in LAT are phosphorylated after stimulation of NK cells through the low-affinity Fc receptor for IgG (FcγRIII; also known as CD16) (REF. 33) or the adhesion receptor CD2 (REF. 34), or following contact with NK-sensitive target cells[33]. Moreover, overexpression of LAT in NK cells enhances ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY and NATURAL CYTOTOXICITY[33]. Despite the finding that NK cells of *Lat*-knockout mice seem to have grossly normal function[13], the data indicate that LAT has a role in NK-cell-receptor-mediated signalling. In human NK cells, LAT is constitutively associated with 2B4 (also known as CD244), an activating receptor that recognizes the ligand CD48 on target cells[35,36], and LAT associates with GRB2 and PLC-γ1 following crosslinking of 2B4 (REF. 37). Although it is unclear whether the association between LAT and

SYK-FAMILY KINASES
The second class of cytoplasmic protein tyrosine kinases that have essential roles in the activation of haematopoietic cells — the other classes being the SRC- and TEC-family kinases. This small group consists of ZAP70 (ζ-chain-associated protein kinase of 70 kDa) in T cells, and SYK (spleen tyrosine kinase) in B cells and myeloid cells. After immunoreceptor ligation, SYKs bind through tandem SRC homology 2 (SH2) domains to the phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs). Subsequently, they become phosphorylated and activated by SRC kinases.

TEC-KINASE FAMILY
A third class of protein tyrosine kinases that is required for the activation of haematopoietic cells — the first and second classes being the SRC- and SYK-family kinases. The TEC-family kinase prototypes are ITK (interleukin-2-inducible T-cell kinase) in T cells and BTK (Bruton's tyrosine kinase) in B cells. Among other functions, TEC kinases seem to have an important role in the activation of phospholipase C enzymes after immunoreceptor ligation.

E3 UBIQUITIN LIGASE
Ubiquitin ligases recruit ubiquitin-conjugating enzymes, which attach the small polypeptide ubiquitin to proteins. The polyubiquitylated proteins are then subjected to proteasomal degradation. Polyubiquitylation, followed by proteasomal degradation, is one of the cellular mechanisms to eliminate proteins.

γδ T CELLS
T cells express either a T-cell receptor (TCR) composed of α- and β-subunits (αβ-TCR) or a TCR composed of γ- and δ-subunits (γδ-TCR). Most human T cells (more than 90%) express αβ-TCRs that mainly recognize antigenic peptides bound to conventional MHC class I or II molecules. T cells that express γδ-TCRs are less abundant, and the ligands for these receptors are less well-characterized.

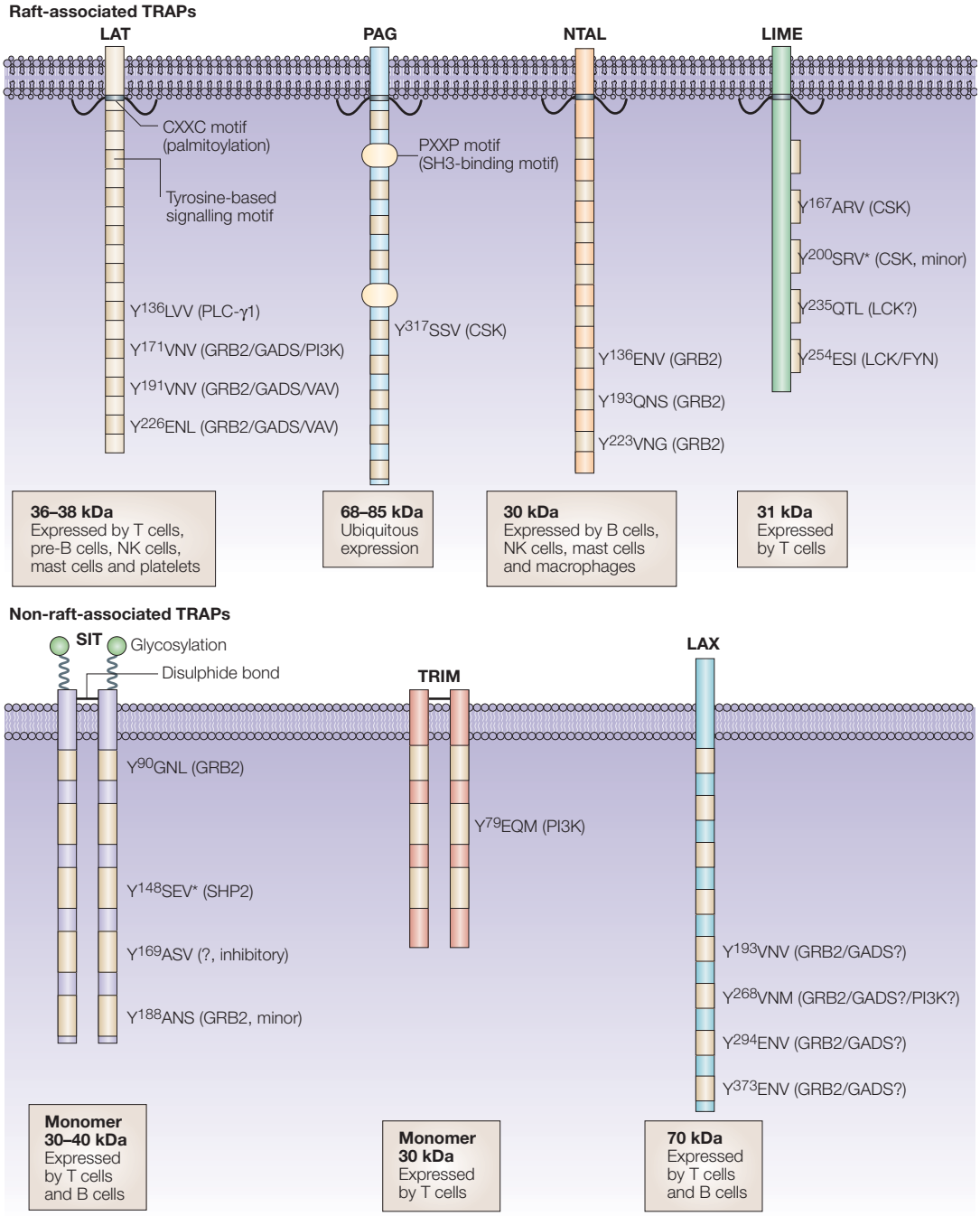


Figure 2 | **The transmembrane adaptor proteins.** So far, seven transmembrane adaptor proteins (TRAPs) have been identified. All of these have short extracellular domains, typical transmembrane α-helices and long cytoplasmic tails that have up to nine potential phosphorylation sites known as tyrosine-based signalling motifs (yellow bars). Four of the known TRAPs localize to lipid rafts — LAT (linker for activation of T cells), PAG (protein associated with glycosphingolipid-enriched microdomains; also known as carboxy-terminal SRC kinase (CSK)-binding protein, CBP), NTAL (non-T-cell activation linker; also known as linker for activation of B cells, LAB) and LIME (LCK-interacting membrane protein). The other three are non-raft proteins — SIT (SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase (SHP2)-interacting TRAP), TRIM (T-cell-receptor-interacting molecule) and LAX (linker for activation of X cells, where X denotes an as yet unidentified cell). All raft-associated TRAPs have a juxtamembrane CXXC motif (where X denotes any amino acid), which becomes palmitoylated and is required to target these proteins to rafts. It has been shown that the CXXC motif is indispensable for the function of LAT[21,96]. Whether this is the case for the other raft-associated TRAPs has not yet been investigated. The phosphorylation status of TRAPs is modulated (mostly increased) after immunoreceptor or co-receptor ligation. The tyrosine-based signalling motifs that are known to become tyrosine phosphorylated and/or known to be involved in recruiting SH2-domain-containing molecules *in vivo* are shown (primary sequences of four amino acids beginning with Y). Potential immunoreceptor tyrosine-based inhibition motifs (ITIMS) in the cytoplasmic domains of the TRAPs are also indicated (*). GADS, GRB2-related adaptor protein; GRB2, growth-factor-receptor-bound protein 2; NK, natural killer; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C.

SH3 DOMAIN
A protein domain that is common in signal-transduction molecules. It interacts specifically with certain proline-containing peptides. Classically, it contains either (R/K)XXPXXP or PXXPXR motifs, where X denotes any amino acid.

2B4 is direct or is mediated indirectly through lipid rafts, the data indicate that 2B4 might mediate NK-cell activation through a LAT-dependent signalling pathway.

Signalling through the high-affinity receptor for IgE (FcεRI) is markedly reduced in LAT-deficient mast cells[38]. However, in contrast to the situation in T cells, the block in FcεRI-mediated signalling in LAT-deficient mast cells is not complete. So, LAT-deficient mast cells are only partially defective in FcεRI-mediated degranulation and cytokine production and can still flux calcium after ligation of FcεRI. This indicates that mast cells have alternative, LAT-independent signalling pathways that regulate calcium flux, degranulation and cytokine release. One candidate molecule that could allow FcεRI-mediated signalling in the absence of LAT is NTAL, which is co-expressed with LAT in mast cells (discussed later).

LAT is also inducibly phosphorylated during the activation of platelets through CD47 (also known as integrin-associated signal transducer)[39], FcγRIIa (also

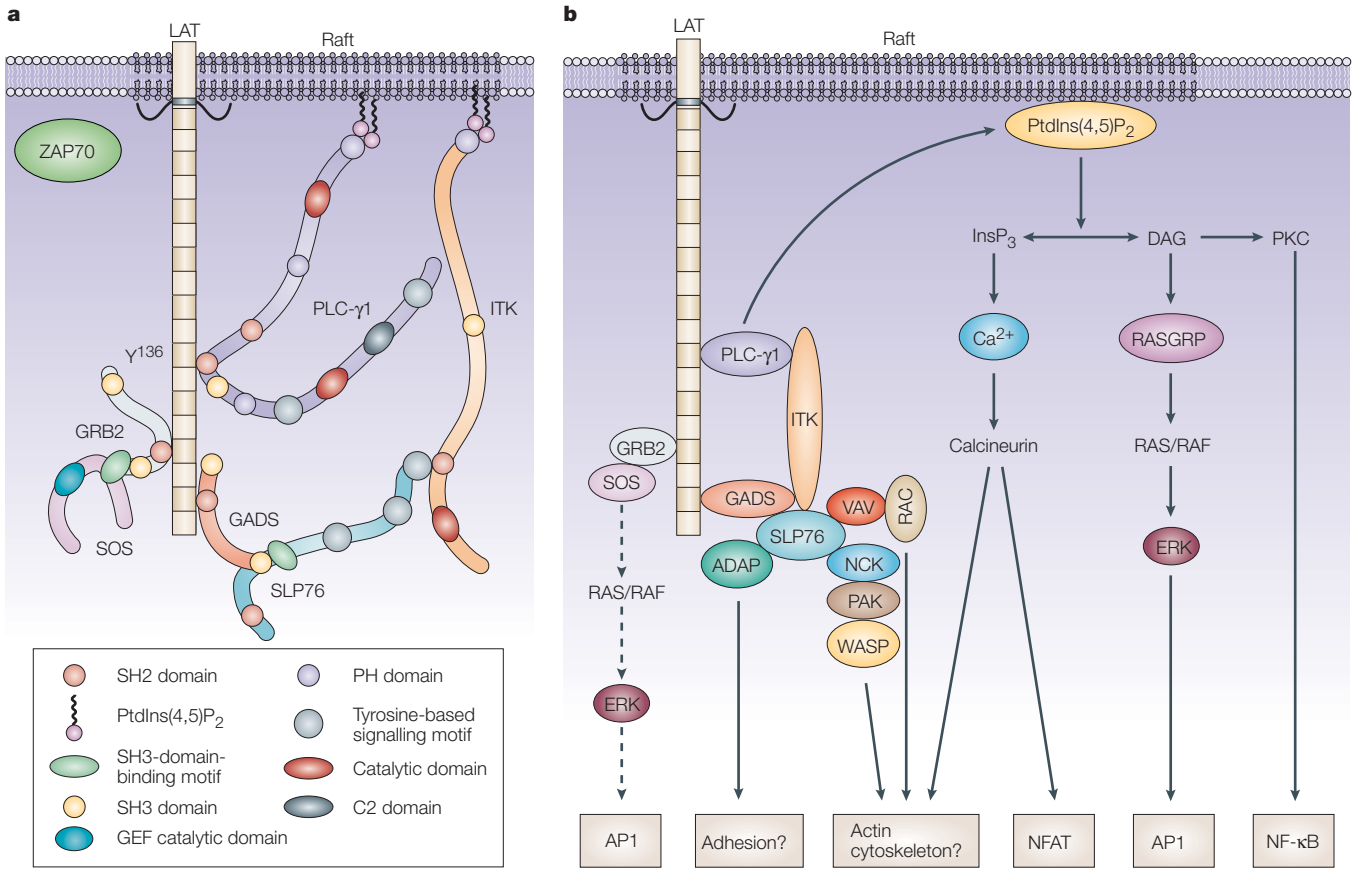


Figure 3 | **The signalosome organized by LAT. a** | LAT (linker for activation of T cells) is a substrate of the protein tyrosine kinase ZAP70 (ζ-chain-associated protein kinase of 70 kDa) in T cells and of SYK (spleen tyrosine kinase) in non-T cells. Following phosphorylation, LAT recruits the GRB2 (growth-factor-receptor-bound protein 2)–SOS (son of sevenless homologue) complex, which is involved in activating the RAS–ERK (extracellular-signal-regulated kinase) signalling pathway. The adaptor protein GADS (GRB2-related adaptor protein) binds to LAT through its SRC homology 2 (SH2) domain and associates through its SH3 DOMAIN with the adaptor protein SLP76 (SH2-domain-containing leukocyte protein of 76 kDa). Phospholipase C-γ1 (PLC-γ1) binds to LAT through its SH2 domain. When bound to LAT, PLC-γ1 and SLP76 undergo ZAP70-mediated phosphorylation. Phosphorylated SLP76 recruits the TEC-kinase ITK (interleukin-2-inducible T-cell kinase) through an SH2-domain-mediated interaction. ITK phosphorylates PLC-γ1, thereby activating the enzyme. This figure is modified with permission from REF. 97 © (2002) American Society for Clinical Investigation. **b** | LAT-associated PLC-γ1 hydrolyses phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) to generate inositoltrisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ induces an increase in the cytoplasmic calcium (Ca^{2+}) concentration, thereby activating the transcription factor NFAT (nuclear factor of activated T cells) through the serine phosphatase calcineurin. DAG activates protein kinase C (PKC) isozymes, most notably PKC-θ, which is involved in activation of the transcription factor nuclear factor-κB (NF-κB) (among other activities). DAG also recruits the nucleotide-exchange factor RASGRP (RAS guanyl-releasing protein), thereby leading to the activation of the RAS/RAF–ERK pathway, which initiates transcriptional activity of activator protein 1 (AP1). The relative contributions of the LAT–GRB2–SOS–RAS and the LAT–PLC-γ1–DAG–RASGRP–RAS pathways for the activation of ERK are not fully elucidated, but in T cells, the latter pathway is probably more important than the former. LAT-bound SLP76 is not only indispensable for the activation of PLC-γ1 but also interacts with several other signalling molecules, such as NCK (non-catalytic region of tyrosine kinase)[98], VAV[99] and ADAP (adhesion and degranulation-promoting adaptor protein)[100], thereby regulating alterations of the actin cytoskeleton after TCR ligation and possibly also regulating cellular adhesion[101,102]. C2 domain, PKC conserved region 2 domain; GEF, guanine-nucleotide exchange factor; NFAT, nuclear factor of activated T cells; PAK, p21-activating kinase; PH, pleckstrin homology; WASP, Wiskott–Aldrich syndrome protein.

ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC). A mechanism by which natural killer (NK) cells kill other cells: for example, virus-infected target cells that are coated with antibodies. The Fc portions of the coating antibodies interact with the Fc receptors (FcγRIII; also known as CD16) that are expressed by NK cells, thereby initiating a signalling cascade that results in the release of cytotoxic granules (containing perforin and granzyme B), which induce apoptosis of the antibody-coated cell.

NATURAL CYTOTOXICITY
Denotes the ability of some cells — mainly natural killer (NK) cells and NKT cells — to kill tumour cells or virus-infected cells after coming into contact with them, without previous deliberate immunization of the host or activation of these immune cells, processes which are required for similar cytotoxic killing by antigen-specific T cells.

RNA INTERFERENCE
(RNAi). A phenomenon in which the expression of a gene is inhibited when a double-stranded complementary RNA molecule is introduced into the organism.

known as CD32) (REF. 40), glycoprotein VI (GPVI; a collagen receptor) or glycoprotein Ib (GPIb; the receptor for von Willebrand factor)[41,42], indicating that it might be involved in signalling through these receptors. However, LAT deficiency has only mild effects on platelet function. In contrast to mice deficient for the cytosolic adaptor protein SLP76 (a crucial downstream partner of LAT) (discussed earlier), which have a severe bleeding disorder, LAT-deficient mice develop no haemorrhage. Although LAT-deficient platelets have a defective response to collagen-related peptide (an agonist for GPVI), they clot normally at high concentrations of collagen, whereas SLP76-deficient platelets do not[42–44]. This indicates that LAT has a less important role than SLP76 in GPVI-mediated platelet activation and haemostasis. It is possible that other LAT-like protein(s) that can recruit SLP76 to the plasma membrane are expressed by platelets and allow signalling through GPVI or other platelet receptors. Again, NTAL is a strong candidate.

Although it was initially assumed that LAT was not expressed in B-lineage cells, it was recently found to have an important role in signalling through the pre-BCR. In contrast to SLP76-deficient T cells, the development of which is completely arrested at the DN3 stage of thymic development[15,16], mice deficient in the B-cell homologue of SLP76, SLP65 (also known as B-cell linker, BLNK), have only a partial block in B-cell maturation at the pre-B-cell stage[45–48]. This indicates the involvement of other, SLP65-independent signalling pathways in pre-B cells. Surprisingly, during the search for such pathways, it was found that pre-B cells express both LAT and SLP76 (REFS 49,50). Biochemical analysis further revealed that in these cells, LAT constitutively interacts with SLP76 and inducibly associates with Igα (also known as CD79a) and PLC-γ2 (REF. 49). Although the molecular mechanisms of how this complex is formed are as yet unclear, the data indicate that there are two signalling modules that link the BCR to calcium signalling in pre-B cells: a SLP65–Bruton's tyrosine kinase (BTK)–PLC-γ2 module and SLP76–LAT–BTK–PLC-γ2 module[49]. Accordingly, LAT-deficient mice were shown to have increased numbers of pre-B cells, indicating a partial block in B-cell development. Furthermore, in contrast to mice deficient in SLP65 alone, B-lineage cells of mice deficient in both LAT and SLP65 were found to be arrested at the pre-B-cell stage[49]. So, LAT is not only involved in the development of T cells but also in the maturation of pre-B cells. It remains to be determined whether concomitant deletion of SLP65 and SLP76 produces an identical phenotype to that of mice deficient in both LAT and SLP65, as would be expected.

NTAL: a LAT-like protein in non-T cells

Similar to the TCR, ligation of the BCR induces PLC-γ activation, calcium flux and ERK activation. However, LAT is not expressed by mature B cells, and therefore, many groups have been searching for a LAT-like molecule that can connect the BCR to intracellular signalling pathways. One candidate was identified recently and is known as NTAL or LAB[51,52]. NTAL is mainly expressed by non-T cells (B cells, NK cells and myeloid cells), and similar to LAT, it is palmitoylated on a juxtamembrane CXXC motif and thereby targeted to lipid rafts. The cytoplasmic domain of NTAL contains eight conserved tyrosine-based signalling motifs (FIG. 2). After BCR or Fc-receptor ligation, NTAL becomes tyrosine phosphorylated and associates with GRB2, GAB1 and CBL, but not with PLC-γ or SLP molecules[51,52].

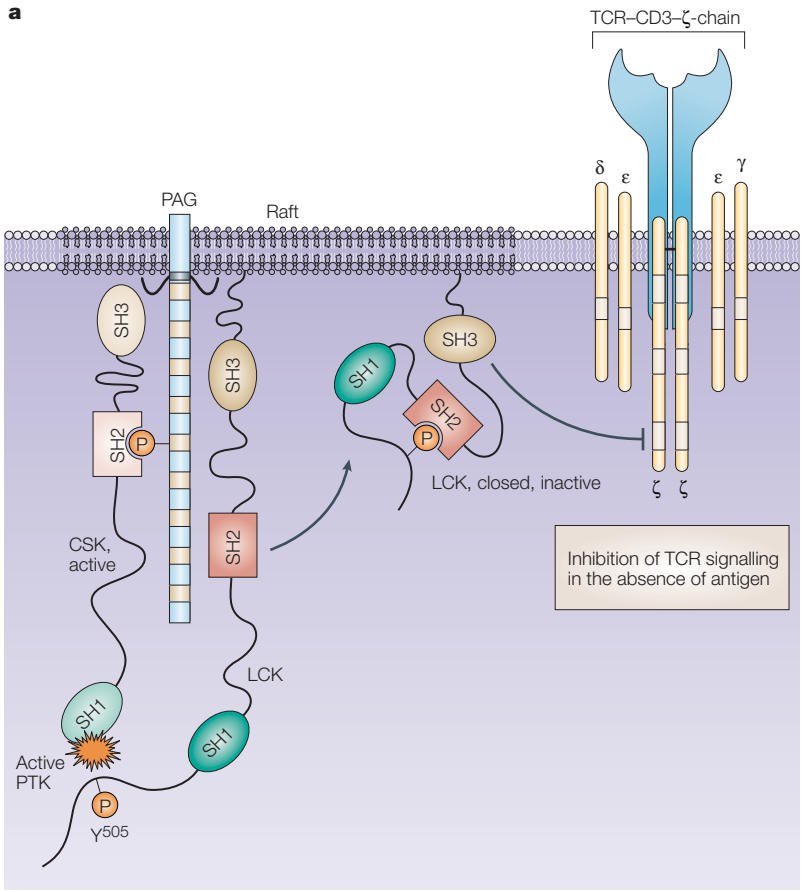
Expression of NTAL in LAT-deficient Jurkat cells can rescue particular aspects of TCR-mediated signalling: either TCR-mediated calcium flux or ERK activation, depending on the experimental system used[51,52]. Similarly, retroviral expression of NTAL in LAT-deficient mice partially rescues the development of mature peripheral T cells, and in this case, the three membrane-distal tyrosine residues seem to be crucial[52,53]. These data indicate that NTAL can replace LAT to some extent. However, transgenic mice that expresss NTAL on a *Lat*$^{-/-}$ background develop an autoimmune phenotype that is almost identical to the phenotype of LAT^{Y136F}-mutant mice[54]. So, in this experimental system, NTAL is functionally equivalent to the LAT^{Y136F} mutant that does not bind PLC-γ1.

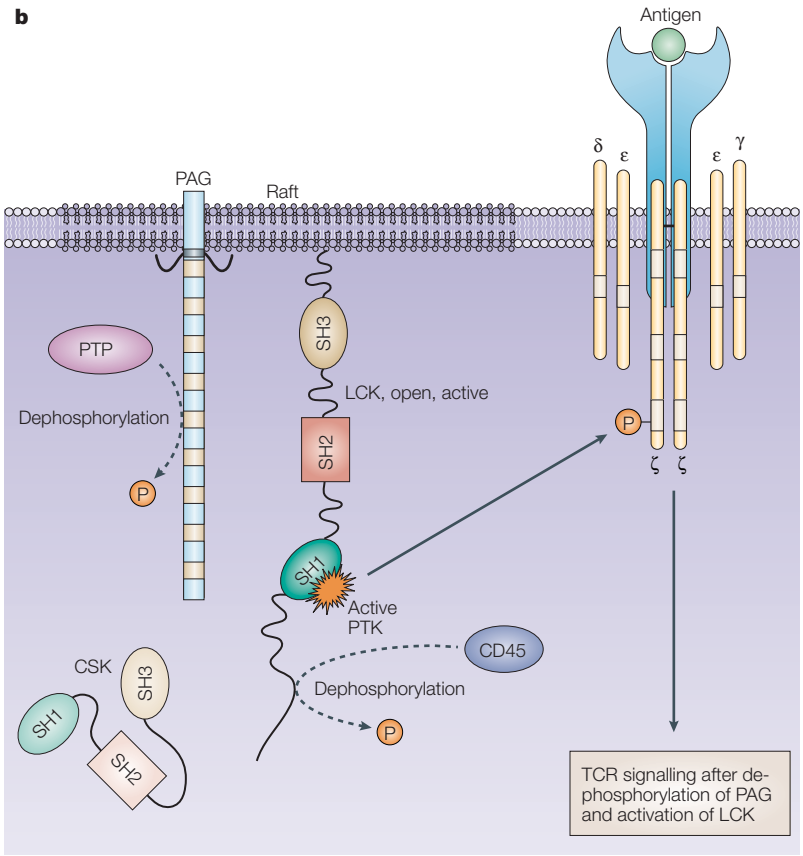
Although NTAL and LAT seem to have overlapping functions, it is not clear whether NTAL functions as a LAT equivalent in B cells. Indeed, because NTAL cannot bind PLC-γ enzymes or SLP-family molecules, it is unlikely that it can mediate efficient induction of BCR-induced calcium flux. This leaves unanswered the question of whether the BCR uses a LAT-like molecule to activate the calcium pathway. Recent data indicate that SLP65 directly associates with both phosphorylated Igα and Igβ through its SH2 domain[55,56]. This could be a B-cell LAT/NTAL-independent mechanism to recruit PLC-γ2 to the BCR for activation of calcium mobilization. However, it is still possible that mature B cells express an as yet unidentified adaptor protein other than NTAL.

NTAL also becomes heavily phosphorylated after ligation of the high-affinity IgG receptor (FcγRI; also known as CD64) on the cell surface of monocytes or FcεRI on mast cells[51]. In addition, a recent report showed phosphorylation of NTAL after ligation of the stem-cell-factor (SCF) receptor KIT (also known as CD117) (REF. 57). Moreover, concomitant downregulation of NTAL and LAT by RNA INTERFERENCE (RNAi) results in a more severe reduction in FcεRI- and KIT-mediated mast-cell degranulation than RNAi-mediated downregulation of either molecule alone[57]. The latter finding indicates that NTAL and LAT cooperate in receptor-mediated mast-cell degranulation. However, the precise mechanisms underlying this cooperation need to be elucidated.

PAG: an inhibitor of SRC kinases

Although it is also found in rafts, PAG (also known as CBP) seems to be functionally distinct from LAT and NTAL[58,59]. PAG is expressed not only by lymphocytes but almost ubiquitously, and its tyrosine motifs are phosphorylated by SRC-family kinases. The cytoplasmic domain contains nine potential tyrosine-phosphorylation sites, but so far, the only binding





partner that has been clearly identified is the cytoplasmic PTK CSK — the most important negative regulator of SRC-family kinases. According to the current model, phosphorylated PAG functions as a membrane anchor for CSK, thereby bringing the kinase in close proximity to its substrates (the SRC-family kinases) that are localized in lipid rafts, to downregulate their enzymatic activities[58,59] (FIG. 4a).

In resting αβ T cells, a high steady-state level of PAG phosphorylation is maintained, most probably by the constitutive activity of the SRC kinase FYN[60]. FYN localizes to rafts and non-covalently interacts with PAG through an unknown mechanism[58]. PAG is the most heavily tyrosine-phosphorylated membrane molecule present in resting αβ T cells[58]. However, immediatedly after stimulation through the TCR, PAG becomes rapidly dephosphorylated[58,61–63], which results in the release of CSK from the membrane (FIG. 4b). This mechanism is thought to relieve the CSK-mediated inhibition of SRC kinases during the initial phase of T-cell activation. The identity of the phosphatase responsible for this activation-linked PAG dephosphorylation is still under debate — CD45 (REF. 61) or SHP2 (REF. 64) seem to be the most probable candidates. Definitive identification of the PAG phosphatase is important, because its dysregulation (causing hyperactivity) could be involved in the development of T-cell-mediated autoimmunity.

Because SRC-family kinases phosphorylate PAG in resting T cells (which leads to the recruitment of CSK and, in turn, the inhibition of SRC kinases), the

Figure 4 | **The role of the PAG–CSK complex in TCR-mediated signalling. a** | In resting T cells, PAG (protein associated with glycosphingolipid-enriched microdomains) is expressed as a tyrosine-phosphorylated protein, which thereby recruits the protein tyrosine kinase (PTK) CSK (carboxy (C)-terminal SRC kinase) to the plasma membrane, through a single tyrosine-based signalling motif. CSK phosphorylates the negative regulatory C-terminal tyrosine residue of the SRC kinase LCK, which subsequently binds to its own SRC homology 2 (SH2) domain. This intramolecular interaction between the SH2 domain and the C-terminal tyrosine residue generates a 'closed' and enzymatically inactive conformation of the kinase. Through this mechanism, PAG is thought to inhibit T-cell receptor (TCR)-mediated signalling in the absence of antigen. **b** | After TCR ligation, PAG becomes dephosphorylated by a protein tyrosine phosphatase (PTP); possible candidates are CD45 and SHP2 (SH2-domain-containing PTP). This releases CSK from the membrane, allowing the SRC kinases, such as LCK, to become activated and TCR signalling to ensue. In pathophysiological conditions, a dysregulated PTP could act on PAG, inducing its partial dephosphorylation. Consequently, the activity of SRC kinases would become upregulated, thereby lowering the thresholds required for T-cell activation. In turn, T cells could become activated, either in the absence of antigen or by ligands that would usually induce tolerance or anergy (partial agonists or antagonists, respectively). These mechanisms could be involved in the generation of autoimmune diseases. It should be noted that PAG has nine potential tyrosine phosphorylation sites, but only the CSK-binding site has so far been shown to become phosphorylated *in vivo*. Whether PAG has additional binding partners is therefore unknown.

PAG–CSK–SRC-kinase–phosphatase system is an interesting model of a negative-feedback loop. The activation of CSK after binding to PAG is seemingly not only due to a 'topological' effect (through proximity to its substrates) but also probably due to an allosteric activation of CSK within the PAG–CSK complex (because binding to PAG seems to induce a conformational change in CSK that increases enzymatic activity)[65]. This regulatory system is fine-tuned by interactions with protein kinase A (PKA). PKA increases the activity of PAG-bound CSK by phosphorylating a crucial serine residue and thereby further contributes to the PAG–CSK-mediated inhibition of signalling through the TCR[66].

PAG also functions as a negative regulator of immunoreceptor signalling in mast cells but by a different mechanism than it uses in T cells. Aggregation of FcεRI is accompanied by rapid phosphorylation of PAG and increased recruitment of CSK[67]. This recruitment of CSK to PAG suppresses the activity of LYN, which mitigates the intensity of FcεRI-mediated signalling processes and impairs FcεRI-induced activation of LYN and calcium flux. The final consequence of this feedback loop is the inhibition of FcεRI-mediated mast-cell degranulation.

Crosslinking of the BCR on mature B cells also induces an increase in PAG phosphorylation and CSK association[68], thereby presumably suppressing the activity of LYN and other B-cell SRC kinases. This might have different effects on the outcome of signalling through the BCR. Indeed, although LYN is required for the initiation of BCR-signalling cascades, through the phosphorylation of ITAMs in the Igα and Igβ chains, it also phosphorylates immunoreceptor tyrosine-based inhibititory motifs (ITIMs) of negative-signalling regulators such as CD22 or paired immunoglobulin-like receptor B (PIRB)[69,70]. So, suppression of SRC-family-kinase activity by BCR-induced PAG phosphorylation (and thereby CSK activation) might help to downregulate B-cell activation or, alternatively, temporarily suppress inhibitory signals that are mediated by ITIM-containing molecules. It could be speculated that the compartmentation of subsets of the SRC-kinase family in raft and non-raft microdomains has an important role in this complex system of feedback loops.

Dysregulation of the PAG–CSK complex substantially contributes to the transformation of cattle lymphocytes by the protozoal parasite *Theileria parva*[71]. The parasite suppresses the expression of PAG by an unknown mechanism, which results in increased kinase activity of haematopoietic-cell kinase (HCK), a SRC-family kinase that is involved in proliferation and induction of a leukaemia-like phenotype of the infected cells. Killing of the parasite by chemotherapy results in re-expression of PAG, recruitment of CSK to rafts and suppression of HCK activity. This mechanism, at least in part, probably causes the loss of the transformed phenotype in cured animals. So, in this case, and perhaps also more generally, the PAG–CSK complex functions as a type of tumour suppressor that helps to inhibit the potentially oncogenic effects of activated SRC kinases. However, it should be noted that PAG is not the only transmembrane protein that can bind CSK — the transmembrane adaptors LIME and SIT, which also bind CSK, are discussed later.

In addition to its CSK-binding function, PAG also seems to mediate crosstalk between lipid rafts and the actin cytoskeleton. This is mediated through the C-terminus of PAG, which binds to a PDZ DOMAIN of the cytoplasmic adaptor protein EBP50 (ezrin, radixin, moesin (ERM)-binding protein 50) (REFS 62,72). In turn, EBP50 interacts through its C-terminal domain with ERM PROTEINS, which are known to bind to F-actin. So, through this EBP50–ERM–F-actin pathway, PAG might provide a link between lipid rafts and the actin cytoskeleton. T-cell activation then leads to loss of this association, allowing the rafts to migrate to the IMMUNE SYNAPSE. Indeed, overexpression of PAG reduces raft mobility and inhibits immune-synapse formation[62]. This might be another mechanism by which PAG regulates T-cell activation.

Experiments using CSK- and PAG-deficient epithelial cells have also revealed a role for this TRAP in cell adhesion. In the absence of CSK, continuous hyperactivation of SRC-family kinases leads to constitutive hyperphosphoryation of PAG, which is accompanied by impaired cell spreading and cell migration on fibronectin-coated surfaces. Similarly, silencing of PAG by RNAi also causes defective cell spreading[73].

LIME: a second TRAP regulating SRC kinases

The most recently discovered raft-associated TRAP is known as LIME[74,75]. It is expressed by T cells and differs from other known TRAPS because it seems to become phosphorylated only after ligation of the co-receptors CD4 or CD8 by antibodies or HIV glycoprotein 120 (gp120) (REF. 74). Following CD4-mediated phosphorylation by SRC-family kinases, LIME simultaneously associates with the SRC-family kinases LCK and FYN and, most importantly, with their negative regulator, CSK[74]. The SRC kinases and CSK are both recruited to LIME through their SH2 domains (FIG. 5). The LIME-associated fraction of LCK is phosphorylated on the C-terminal negative-regulatory tyrosine (most probably by LIME-bound CSK), but somewhat paradoxically, it has increased enzymatic activity compared with the total pool of LCK[74]. This is probably because the phosphorylated C-terminal inhibitory tyrosine of LIME-bound LCK cannot bind (intramolecularly) to its own SH2 domain because the latter is already engaged by phosphorylated LIME. Consequently, the inactive, 'closed' conformation of LCK cannot occur in the LIME–CSK–LCK complex, and therefore, the LIME-bound LCK is maintained in an activated state, despite the presence of its inhibitor, CSK, in the same complex (FIG. 5). In this model, LIME is a positive regulator of TCR-mediated signalling, a view that is supported by the finding that overexpression of LIME in Jurkat T cells amplifies TCR-mediated calcium flux, phosphorylation of ERK and transcriptional activity of the *IL-2*-gene promoter[74,75].

PDZ DOMAIN
A protein domain that is common in signal-transduction molecules. It can interact with different amino-acid motifs that are present at the carboxyl terminus of proteins. PDZ domains can also interact with other PDZ domains, with certain internal peptide sequences and even with lipids.

ERM PROTEINS
Ezrin, radixin, moesin and the closely related merlin are structurally similar cytoplasmic adaptor (linker) proteins that contain an amino-terminal domain able to interact with some membrane-linked proteins or ERM-binding protein 50 (EBP50)-family adaptors. They also have a carboxy-terminal domain that can interact with F-actin. So, the ERM proteins link receptors and other membrane proteins to the actin cytoskeleton.

IMMUNE SYNAPSE
A large junctional structure that is formed at the cell surface between a T cell that is interacting with an antigen-presenting cell. It is also known as the supramolecular activation cluster (SMAC). Important molecules involved in T-cell activation — including the T-cell receptor, numerous signal-transduction molecules and molecular adaptors — accumulate in an orderly manner at this site. Mobilization of the actin cytoskeleton of the cell is required for immune-synapse formation.

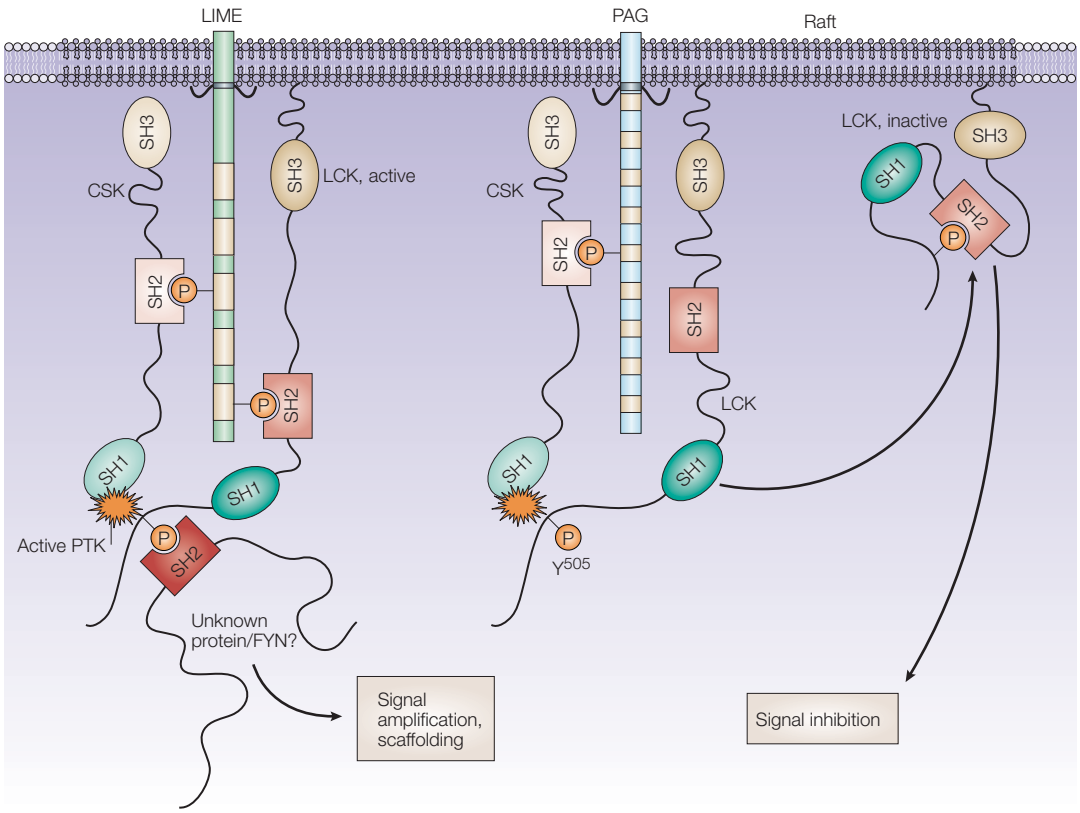


Figure 5 | **Model of LIME- versus PAG-mediated regulation of SRC-kinase activity.** Similar to PAG (protein associated with glycosphingolipid-enriched microdomains), LIME (LCK-interacting membrane protein) is also capable of recruiting CSK (carboxy (C)-terminal SRC kinase) to lipid rafts. However, in marked contrast to PAG, LIME also interacts in a phosphorylation-dependent manner with the SRC homology 2 (SH2) domain of SRC-kinases: that is, LCK and/or FYN in T cells. The blockade of the SH2 domain of the SRC kinase by phosphorylated LIME prevents the induction of the closed conformation, leaving the LIME-associated SRC kinases enzymatically active. So, even in the presence of CSK, LIME could be a positive regulator of SRC kinases. The possibility that the phosphorylated C-terminal tyrosine residue of LIME-associated SRC kinases can bind to another SH2-domain-containing signalling molecule (thereby converting the SRC kinase into a scaffolding protein) needs further analysis. In addition, it needs to be clarified whether PAG and LIME reside within the same or different pools of lipid rafts[103,104]. PTK, protein tyrosine kinase.

One question that arises from this model is what happens to the phosphorylated inhibitory C-terminal tyrosine residue of LCK, which in this situation is not engaged (FIG. 5). Provided that it does not become immediately dephosphorylated by a tyrosine phosphatase, it could interact with another SH2-domain-containing signalling molecule. It is tempting to speculate that LIME could be the missing link in the recently described sequential activation of LCK and FYN that occurs in the lipid rafts of primary T cells[76–78]. Co-engagement of CD4 and the TCR seems to lead to transient shuttling of LCK from non-raft membrane regions to lipid rafts, which is followed by, and required for, the activation of FYN[76]. Although the functional roles of FYN during T-cell activation are still not well defined, it is intriguing to speculate that LIME provides the 'raft anchor' for the shuttling pool of LCK; the phosphorylated C-terminal tyrosine of LIME-bound LCK could then bind the SH2 domain of FYN, thereby bringing FYN into the complex and allowing its activation. After some time, the phosphorylated LCK would dissociate from LIME (because the interactions of the SH2 domains with their ligands are typically reversible and of medium affinity), take up the closed conformation and remain inactive in the lipid raft.

Although the currently available data (which were obtained exclusively using Jurkat T cells) indicate that LIME mainly has a positive regulatory role in TCR-mediated signal transduction, the heavy tyrosine phosphorylation of LIME after CD4 crosslinking could also indicate that LIME is involved in mediating the inhibitory signals that are induced by the crosslinking of CD4 in the absence of additional signals through the TCR–CD3 complex. It is well known that both antibodies specific for particular CD4 epitopes and the binding of HIV gp120 inhibit or markedly modify the outcome of subsequent signalling induced by TCR ligation[79,80]. Indeed, the crosslinking of CD4 *in vivo* by certain antibodies induces a state of antigen-specific tolerance that is due to the development of regulatory T cells, which secrete suppressive cytokines[81]. It is not known whether LIME has a role in T_{Reg}-cell-mediated inhibition of immune responses, but this is an intriguing possibility.

In addition to the previously discussed mechanism, LIME might have a role in the downregulation of lymphocyte function-associated antigen 1 (LFA1)-dependent T-cell adhesion after the crosslinking of CD4 (REF. 82). So far, little is known about the molecular mechanisms that underly the inhibitory functions of CD4. However, because LIME is the main protein that is phosphorylated after CD4 crosslinking, it might sequester some crucial fraction of LCK from CD4 and, at the same time, bring more CSK to membrane rafts, subsequently inhibiting the SRC-family kinases that are required for signalling through the TCR. In this regard, it is interesting to note that CD4-specific antibody treatment was shown to increase the activity of the CSK that is bound to PAG[83] — therefore, there might be some cooperation between the two TRAPs (LIME and PAG) that occupy the same raft environment.

One could also speculate that because LIME has an unusually basic cytoplasmic domain (similar to the ζ-chain of the TCR–CD3 complex[84]), it might have inhibitory-signalling functions in resting, non-stimulated T cells, through interaction with negatively charged phospholipids in the cytoplasmic leaflet of the plasma membrane. By this mechanism, LIME could prevent the interactions of the phospholipids with other important signalling molecules, such as PKC isoenzymes or phosphatidylinositol 3-kinase (PI3K). The phosphorylation of LIME that is induced by CD4 or CD8 crosslinking reduces its positive charge and thereby relieves its inhibitory effect on membrane phospholipids. Such a mechanism might contribute to the setting of inhibitory thresholds on signalling in resting T cells and to the lowering of these thresholds during the initial phases of TCR–co-receptor signalling.

The non-raft TRAPs: TRIM, SIT and LAX

The three other TRAPs known to be involved in immunoreceptor signalling are TRIM, SIT and LAX (FIG. 2). These proteins are not associated with membrane rafts because they do not have the juxtamembrane palmitoylation motif, CXXC. Here, we summarize our limited knowledge of the functions of the non-raft-associated TRAPs.

SIT. In contrast to the other known TRAPs, SIT is unique in being an N-glycosylated molecule. The glycosylation occurs on a single asparagine residue in the extracellular domain, and the N-linked carbohydrate chains might be binding sites for as yet unidentified extracellular lectin ligand(s)[85]. The cytoplasmic domain of SIT contains five tyrosine residues, which are potentially phosphorylated by the concerted activities of SRC- and SYK-family kinases following TCR crosslinking, thereby allowing the binding of the cytoplasmic adaptor GRB2 and the phosphatase SHP2 (REF. 85). In addition, similar to PAG and LIME, phosphorylated SIT can bind to the cytoplasmic PTK CSK[86]. So, because of the inducible binding of the potentially inhibitory signalling molecules SHP2 and CSK, SIT could be involved in the negative regulation of TCR-mediated signalling. This idea is supported by overexpression studies in Jurkat T cells, which revealed an inhibitory role for SIT in TCR-mediated activation of the transcription factor NFAT[85,86]. The mechanism by which SIT impairs TCR signalling in the Jurkat system is as yet unclear but seems to be mediated exclusively through a single tyrosine-based signalling motif, Y^{169}ASV (REF. 86).

TRIM. TRIM was initially identified as a molecule that specifically associates with the TCR–CD3–ζ-chain complex[8]. TRIM is structurally similar to SIT in that it forms a disulphide-linked homodimer and becomes phosphorylated by SRC-family tyrosine kinases in response to T-cell activation. The cytoplasmic domain of TRIM contains three tyrosine-phosphorylation motifs. With the exception of the p85 regulatory subunit of PI3K[8], the physiological binding partners of TRIM are unknown.

Overexpression of TRIM in Jurkat T cells was shown to increase the number of TCR complexes displayed on the cell surface, which correlated with the ability of the TCR to induce calcium flux[87]. So, TRIM might be involved in regulating TCR-mediated signalling by altering the concentration of cell-surface TCR molecules. Experiments based on TRIM overexpression in Jurkat cells indicated that this is probably mediated by interference with spontaneous TCR internalization[87]. Therefore, TRIM-deficient T cells would be expected to have lower cell-surface expression levels and higher TCR-internalization rates, thereby impairing TCR-mediated signalling. However, preliminary characterization of TRIM-deficient T cells indicates that the main TCR-mediated signalling pathways are not impaired (B.S., L. Simeoni and U. Kölsch, unpublished observations). So, the role of TRIM in T-cell activation and development remains unclear.

LAX. The search for a non-T-cell homologue of LAT led to the identification not only of NTAL but also of an additional non-raft TRAP known as LAX[88]. LAX is expressed by T cells, B cells and other haematopoietic cells. The cytoplasmic domain of LAX contains eight tyrosine residues (FIG. 2), of which the four membrane-distal residues are binding sites for GRB2, GADS and PI3K[88]. Similar to LAT, LAX becomes phosphorylated by SRC- and SYK-family tyrosine kinases after T-cell activation, but so far, its role in regulating T-cell activation is unclear. Even though LAX has tyrosine-based signalling motifs similar to those of LAT, LAX fails to reconstitute TCR-mediated signalling in LAT-deficient cells[88]. Instead, overexpression of LAX in Jurkat T cells inhibits the TCR-mediated activation of p38 mitogen-activated protein kinase (MAPK), but not the activation of ERK and JUN amino-terminal kinase (JNK). It is not clear how LAX mediates the inhibition of p38 MAPK. However, because it has several GRB2- and GADS-binding sites, LAX could sequester GRB2 and GADS from LAT, thereby limiting the activation of the MAPK pathway. The finding that the expression of LAX is upregulated rapidly after T-cell stimulation supports this hypothesis[88]. However, further studies are required to determine the precise function of LAX in T-cell activation.

Concluding remarks

It is clear that, at least in T cells, LAT is a key molecule in the organization of immunoreceptor-mediated signalling. The functions of the other known TRAPs are less well understood at present, mainly because data from gene-knockout models are not yet available. A plausible hypothesis is that they are used by haematopoietic cells to finely adjust cellular responses: for example, in particular environments and/or under particular physiological and/or pathophysiological conditions. Alterations in the delicate balance of signals that are mediated by positive and negative regulatory TRAPs could have severe consequences for immune homeostasis *in vivo*, such as the development of autoimmunity, immunodeficiency or cancer.

At present, it is also not clear why some TRAPs are found in lipid rafts, whereas others are non-raft proteins. This is part of a broader question — why do some signalling events occur mainly in rafts, whereas others occur outside them? The targeting of LAT, PAG and LIME to rafts is understandable, as these molecules organize 'signalosomes' in this subcellular compartment. By contrast, the exclusion of TRIM, SIT and LAX from rafts is more difficult to explain. However, other structurally similar and functionally important signalling components of immunoreceptors — the ζ- and γ-chains (BOX 1) — are also non-raft molecules. So, the non-raft-associated TRAPs could be subtle amplifiers of immunoreceptor-mediated signalling. Alternatively, they could be involved in upregulating signalling thresholds: for example, by sequestering components of the signalling machinery away from the lipid rafts. The overexpression experiments that indicated that SIT and LAX mainly have negative regulatory roles (discussed earlier) support this idea.

A comparison of the tyrosine-based signalling motifs of the various TRAPs indicates a high degree of redundancy in this group of signalling molecules (FIG. 2). This might prevent the easy assignment of particular functions to those TRAPs that are not crucial for immunoreceptor-mediated cellular activation. The more subtle functions exerted by these TRAPs might be difficult to detect using conventional screening assays. Furthermore, all TRAPs are not necessarily exclusively regulated by immunoreceptors. Instead, their phosphorylation patterns might be influenced by signals from other receptor–ligand interactions on the surface of haematopoietic cells, as has been shown for LIME[74].

In the next few years, it is possible that some of these questions about the functions of TRAPs will be answered using knockout mice, or transgenic animals that overexpress TRAPs or TRAP mutants. Indeed, preliminary analysis of SIT-deficient mice supports the idea that SIT is a negative regulator of T-cell activation (B.S. and L. Simeoni, unpublished observations). Furthermore, analysis of mice deficient in both SIT and TRIM might clarify whether these structurally similar TRAPs (FIG. 2) can substitute for each other, and the analysis of mice deficient in PAG and/or LIME might clarify the roles of these two CSK-binding TRAPs in regulating SRC-kinase activity in lipid rafts.

A final question concerns whether all of the TRAPs have been identified. Database searches using particular algorithms might lead to the identification of new members of this emerging group of signalling proteins. For example, it is clear that for a protein to be associated with rafts, it does not require a transmembrane region. So, it is possible that there are non-transmembrane adaptors containing several tyrosine-based signalling motifs that are targeted to lipid rafts simply by fatty-acid modifications. In summary, there is still much to discover about TRAPs and their relatives and how these molecules finely tune cellular activation and differentiation processes in the immune system.

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Acknowledgements
This work was supported by grants from the German Research Foundation (DFG) to B.S. V.H. is supported by the Center of Molecular and Cellular Immunology, Ministry of Education, Youth and Sports, Czech Republic. W.Z. is supported by grants from the National Institutes of Heath, United States. We thank M. Kliche and M. Togni for their support during the generation of the artwork, and J. Wienands and J. Lindquist for careful reading of the manuscript.

Competing interests statement
The authors declare no competing financial interests.

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