FEEDBACK REGULATION OF LYMPHOCYTE SIGNALLING

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The development, survival and activation of lymphocytes is controlled by a multitude of extracellular signals in the form of soluble or membrane-bound ligands. Binding of these ligands to receptors on the lymphocyte surface is translated into intracellular signals that are processed in various ways inside the cell and determine its fate. The processing of an incoming signal involves amplification, diversification and termination. Feedback signalling loops have an essential role in the control of these processes, yet our knowledge about these regulatory loops is limited. However, several new feedback regulatory circuits have been recently discovered in lymphocytes and it is probable that more of these circuits will be found in the near future. Here, we give an overview of the present knowledge and working principles of such feedback loops.

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Many intracellular signalling molecules have been identified in recent years and their importance has been recognized by biochemical and loss-of-function studies, such as knockout or mutational screens[1,2]. The information gained by these approaches can be summarized in signalling schemes that show the inter-connectivity of signalling proteins by arrows leading to the nucleus. However, feedback loops that have an essential part in the amplification, as well as attenuation, of intracellular signalling pathways already occurring in the cytosol are rarely shown in such schemes. The reason for this is that feedback loops cannot be easily identified by classical genetic or modern loss-of-function approaches. These methods can order signalling elements in a linear pathway but not within a feedback loop, in which the participating elements are upstream and downstream at the same time. In reality, it is probable that each signalling element in a living cell is part of several feedback loops that control the strength and duration of a signal. As the kinetic parameters of signalling that are regulated by feedback loops have an important role in cell-fate decisions[3], for example, CD4/CD8-lineage commitment[4–6], it is important to learn more about these events. By using specific inhibitors[7,8], by rebuilding part of a signal pathway[9] or by identifying new protein interactions[10] or modifications — for example, phosphorylation mediated by a downstream signalling element[8,11,12] — several feedback loops have been discovered in recent years. This review focuses on the signalling pathways used by antigen receptors on lymphocytes and describes the working principles of feedback loops that involve cytosolic signalling proteins rather than transcription factors for which more information is available.

The reception of an extracellular signal and its processing inside the cell can be ordered into several phases (FIG. 1). In most cases, an extracellular signal (that is, a ligand) is sensed by membrane-bound receptors, which translate the extracellular signal into an intracellular signal[13]. The initiation of an intracellular signal is determined by a critical threshold, which might involve negative feedbacks and ensures that a cell responds only to a certain quantity and quality of extracellular signal. Once initiated, an intracellular signal is often amplified by positive-feedback loops and diversified into several signalling routes, which control different effector functions inside the cell. The strength and duration of the signal is also controlled by negative-feedback loops that can terminate an intracellular signal. A special case is a double-negative feedback loop, which is characterized by the inhibition of a signal inhibitor (FIG. 1). The outcome of this regulation is positive and results in strong amplification of the signal[14].

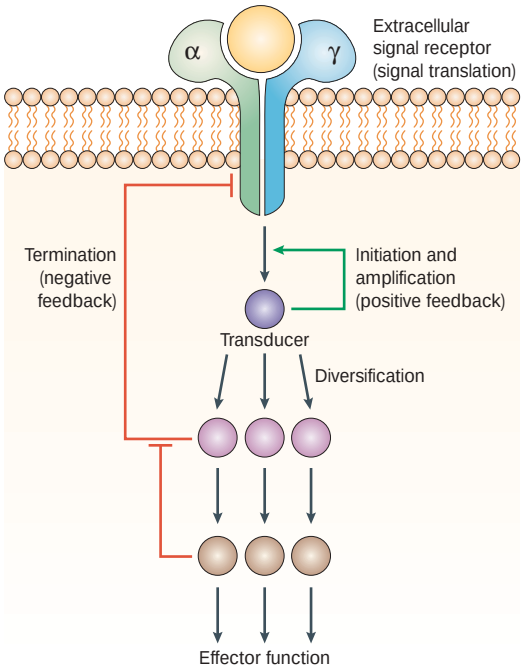

Figure 1 | **Mechanisms of intracellular signal transduction.** After ligand binding, receptors undergo conformational changes that are translated into intracellular signals. The initiation of a signal often involves positive-feedback loops (green arrow) resulting in signal amplification. Signal transducers are multidomain proteins that interact with various signalling elements and mediate signal diversification. Many elements in a signalling pathway initiate negative feedback (red lines), resulting in signal termination. However, negative feedback could also determine the threshold value for receptor activation. An important feedback system is the so-called double-negative feedback system, which is characterized by the inhibition of an inhibitor.

ALLOSTERIC PROTEINS
Proteins that contain two or more topologically distinct binding sites, which interact functionally with each other; for example, binding of a ligand, often a product or downstream effector of the protein, to one site affects the activity or accessibility of the other site.

SRC-HOMOLOGY 2 DOMAIN
(SH2 domain). A protein domain commonly found in signal-transduction molecules that interacts specifically with phosphotyrosine-containing peptides.

SRC-HOMOLOGY 3 DOMAIN
(SH3 domain). A protein domain that binds proline-rich peptide sequences with the consensus Pro-Xaa-Xaa-Pro. The binding between SH3 domains and their target sequence is often regulated by serine/threonine phosphorylation of the target protein close to the recognition site.

PLECKSTRIN-HOMOLOGY DOMAIN
(PH domain). A protein domain that binds to the charged headgroups of specific polyphosphoinositides and thereby targets signalling proteins to specific regions of the plasma membrane enriched by these lipids.

IMMUNORECEPTOR TYROSINE-BASED INHIBITORY MOTIF
(ITIM). This motif is found in the cytoplasmic domains of inhibitory receptors. After ligand binding, the ITIM (Val/Ile-Xaa-Tyr-Xaa-Xaa-Leu/Val) becomes tyrosine phosphorylated, which recruits and activates phosphatases.

IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF
(ITAM). A structural motif containing tyrosine residues, found in the cytoplasmic tails of several activating receptors. The motif contains two tyrosines in the sequence context Tyr-Xaa-Xaa-Leu/Ile. After tyrosine phosphorylation, the motif becomes a binding target for SRC-homology 2-domain-containing proteins.

Regulation of signalling molecules

For a better understanding of the design of feedback loops, it is important to know more about the regulation of the individual signalling elements that constitute such signalling loops. Most signalling proteins are ALLOSTERIC PROTEINS that change their conformation according to their signalling status. Like a switch with multiple settings, signalling proteins can reside in different activation states, between inactive and fully active. The setting of these switch-like molecules is not only determined by input signals, but also by feedback signals. To allow this regulation, most signalling molecules are multidomain proteins that consist of regulatory domains and functional domains, the latter of which can either mediate binding or have catalytic activity[15,16] (FIG. 2a). The 'off' state of these proteins is characterized by an intramolecular interaction, whereby a regulatory domain of the molecule folds back to either block or inhibit the catalytic or binding domain of a signalling element (FIG. 2b). Such autoinhibitory control is not only found in many cytosolic signalling proteins, but is also displayed by several receptors, such as the epidermal growth factor (EGF) or fibroblast growth factor (FGF) receptors[17].

The regulatory domains of signalling molecules often have a dual function. In the 'off' state, they inhibit the activity of the signalling protein and in the 'on' state, they function as a targeting domain, allowing the signalling molecule to bind to its target structure. In this way, a signalling protein becomes fully active only at the appropriate place within the cell, where it encounters its reaction or binding partner. This regulation ensures that signalling occurs in a spatially restricted manner in the cell. An autoinhibitory function has been described for a diverse group of binding domains, such as the SRC-HOMOLOGY 2 (SH2), SRC-HOMOLOGY 3 (SH3) and PLECKSTRIN-HOMOLOGY (PH) domains, which are involved in the binding of phosphotyrosines, proline-rich sequences and phospholipids, respectively[18].

The crystal structure of SH2-domain-containing protein tyrosine phosphatase 2 (SHP2) provides an example of how the autoinhibition of a signalling protein can work[19–21]. In the closed, inactive conformation, the amino-terminal SH2 domain folds back to block the substrate-binding site of the phosphatase domain, thereby preventing phosphatase activity (FIG. 2b). Such autoinhibition is also likely to control the activity of the SHP2-related phosphatase SHP1. SHP1 is an important negative regulator of antigen-receptor signalling in lymphocytes, in which it binds to proteins such as CD22 or CD72 that have an IMMUNORECEPTOR TYROSINE-BASED INHIBITORY MOTIF (ITIM) in their cytoplasmic tails. After lymphocyte activation, the ITIM tyrosines of CD22 become phosphorylated and can be bound by the two SH2 domains of SHP1, thereby locking the enzyme in an open and more active conformation[22]. However, even in its open, active conformation, the catalytic activity of SHP1 can still be regulated by the redox equilibrium in the cell. In the presence of hydrogen peroxide (H_2O_2) or other reactive oxygen species (ROS), an essential cysteine residue in the catalytic site of SHP1 is oxidized and renders the protein tyrosine phosphatase completely inactive[23] (FIG. 2b).

Another example of allosteric regulation of a signalling protein is the protein tyrosine kinase (PTK) SYK, which has an important role in the initiation of signalling from the B-cell receptor (BCR)[24,25]. Although a crystal structure of the complete enzyme is not yet available, the functional analysis of many SYK mutants shows that the carboxy-terminal SH2 domain of SYK inhibits the activity of this kinase[9] (FIG. 2c). SYK is released from autoinhibition only when in contact with its target — that is, a receptor with an IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM). SYK can phosphorylate the two ITAM tyrosines and after binding of its two tandem SH2 domains to the dual phosphorylated ITAM (ppITAM), assumes an open and more active conformation. The full activation of SYK requires not only an open conformation, but also the autophosphorylation of SYK at the regulatory loop of the kinase domain[26] (FIG. 2c). However, in the open and active conformation, SYK also becomes phosphorylated on several other tyrosine residues that are situated in the linker region connecting the two tandem SH2

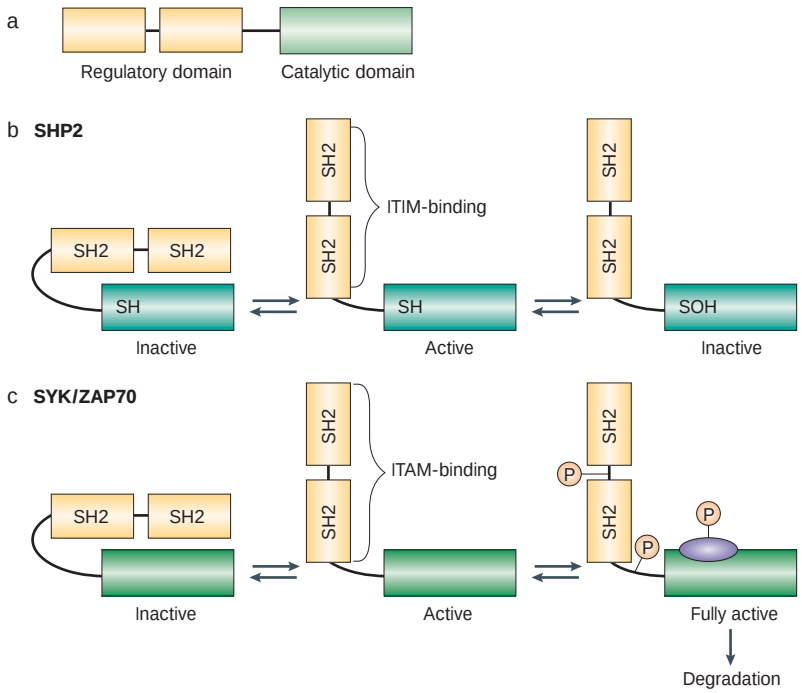

Figure 2 | **Regulation of signalling molecules. a** | Structural organization of a signalling molecule consisting of regulatory and catalytic domains. **b** | Model of regulation of the protein tyrosine phosphatase SHP2. In its inactive state, the two SRC-homology 2 (SH2) domains of SHP2 fold over the catalytic domain and prevent interaction of the catalytic domain with its substrates. Activation is brought about by a conformational change, which renders the catalytic and regulatory domain accessible for interaction with the substrate and binding target, respectively. Inactivation of the open phosphatase can be achieved by reversible oxidation of a cysteine residue in the catalytic domain of SHP2. **c** | Model of the regulation of protein tyrosine kinases (PTKs) of the SYK/ZAP70 family. In their inactive state, the regulatory loop consisting of the tandem SH2 domain is folded over the catalytic domain and prevents interaction with substrate proteins. The regulatory lobe is displaced after activation of the kinase activity, which can be further increased by autophosphorylation of the kinase regulatory loop[26]. The phosphorylation of tyrosines in the linker region between the carboxy-terminal SH2 and kinase domain triggers polyubiquitylation by CBL proteins and removal of activated SYK/ZAP70 molecules by proteasomal degradation[96–101]. ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; SHP2, SH2-domain-containing protein tyrosine phosphatase 2; ZAP70, ζ-chain-associated protein of 70kDa.

domains or the SH2 domains with the kinase domain[27–29]. These phosphorylations can be either activating or inhibitory. For example, the phosphorylation of Tyr317 of SYK by the PTK LYN results in the binding of a CBL-containing ubiquitin-ligase complex to SYK, and its subsequent UBIQUITYLATION and degradation[29,30]. Such negative regulation ensures that highly active signalling proteins have only a short life span.

UBIQUITYLATION
Covalent conjugation of the highly conserved protein ubiquitin to a target protein via an isopeptide bond between its carboxyl terminus and the ε-amino group of a lysine residue of the target protein. Ubiquitylation is often triggered by phosphorylation of the target protein close to the acceptor lysine residue. Ubiquitylation can fix an allosteric protein in a specific conformation or target it for degradation by the proteasome complex.

Feedback regulation of intracellular signalling

The activity of signalling molecules are regulated in various ways by feedback loops. Feedback loops occur in many different forms in the physical and biological world. They can either be positive or negative, or have more complex features that are often seen in bi-stable signalling systems, such as the double-negative feedback loops previously mentioned. In the next section, several examples of how intracellular signals can be amplified by positive-feedback loops are described.

Positive-feedback loops

A good example of positive feedback is a forest fire. Normally, the high threshold set by the ignition temperature of wood (233°C) prevents a fire. However, once a fire has started, a positive-feedback loop (fire–heat–wood–fire) is initiated that rapidly amplifies the range of the fire, as long as wood and oxygen are available. As seen by this example, one characteristic of positive feedback is that it becomes independent of the input signal and that once in full swing, it is hard to control. In cell signalling, positive feedbacks are tightly regulated by critical thresholds and only occur in a spatially restricted manner to limit the level of signal amplification. This is important, as excessive signalling can induce apoptosis or oncogenic transformation of cells. The simplest form of positive feedback is direct enzyme–product feedback. Here, the product of an enzymatic reaction binds to the regulatory domain of the enzyme and increases its catalytic activity. Such a mechanism has an essential role in the initiation and amplification of signalling from the BCR[9]. Ligation of the BCR results in the activation of receptor proximal PTKs, such as SYK and the SRC-family member LYN, and the rapid phosphorylation of several PTK substrate proteins, including the signalling subunit of the BCR, the Ig-α/Ig-β heterodimer. The two tyrosine residues of the ITAMs of Ig-α and Ig-β have a crucial role in the initiation of BCR signalling[31]. It was previously thought that only SRC-family kinases could phosphorylate the ITAM tyrosines. However, recently, it has been found that SYK itself is the main ITAM kinase[9]. This notion is supported by analysis of mutant mice with targeted deletion of the genes encoding all three of the most active SRC-family kinases in B cells[32]. After BCR activation, the B cells from these mice do not show reduced ITAM phosphorylation.

Due to autoinhibition, unbound SYK is not a very active kinase. This regulation ensures that SYK does not phosphorylate random targets in the cytosol. Yet, when in contact with the BCR, SYK can become active and phosphorylate the two ITAM tyrosines in Ig-α (FIG. 3a). The resulting ppITAM is bound with high affinity by the tandem SH2 domains of SYK, which upon binding, is stabilized in an open and more active conformation. The ppITAM binding not only actives SYK, but also localizes it beneath the plasma membrane, where it can rapidly phosphorylate the ITAMs of neighbouring BCR molecules. This phosphorylation results in more SYK recruitment, SYK autophosphorylation and activation, and amplification of the BCR signal. If not stopped by other means (see later), this positive SYK–ppITAM feedback loop of BCR signalling is active, as long as there are unphosphorylated ITAM tyrosines and free SYK available. The importance of this positive feedback for BCR function is indicated by the signal deficiency of cells that have point mutations of either the ITAM tyrosines[33] or the Phe-Leu-Val-Arg motif in the phosphotyrosine-binding site of the carboxy-terminal SH2 domain of SYK[26].

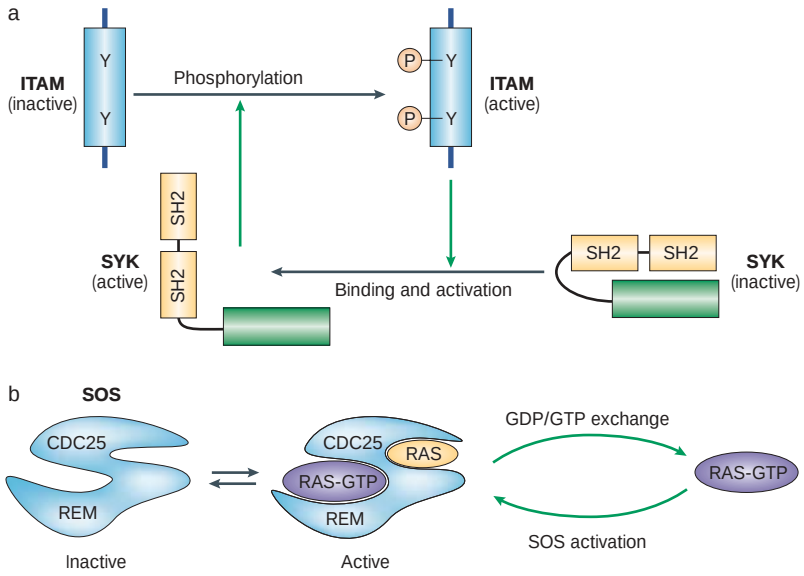

Figure 3 | **Enzyme–substrate positive-feedback loops. a** | The positive-feedback loop involving allosteric activation of the kinase SYK by its product, the phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) of Ig-α. **b** | A positive-feedback loop involving allosteric activation of the GDP/GTP-exchange factor son-of-sevenless (SOS) by its product RAS-GTP. A crystal structure of the SOS–RAS complex showed that SOS can bind two RAS molecules, one nucleotide-free RAS molecule at the CDC25 catalytic domain and one RAS-GTP molecule at the regulatory REM (RAS exchanger motif) domain[43]. In the absence of RAS-GTP, the regulatory domain of SOS partly blocks binding of the SOS catalytic domain to RAS-GDP, and therefore, the exchange reaction. Once SOS has produced some RAS-GTP, this product binds to the SOS regulatory domain, thereby fixing the enzyme in an open and ten-fold more active conformation. By this enzyme–substrate positive-feedback loop, the SOS/RAS signal is rapidly amplified. Furthermore, SOS could also be activated by RAS-GTP produced by other exchange factors, such as RAS-GRP — an important RAS activator in T and B cells[40,42,102]. SH2, SRC-homology 2 domain.

Another example of an enzyme–product positive-feedback loop is the rapid activation of the small G-protein RAS, which binds to and activates several intracellular target proteins, such as serine/threonine kinases of the RAF family and the catalytic subunit of phosphatidylinositol 3-kinase (PI3K)[34–36]. The activation of RAS effectors has a pivotal role in lymphocyte signalling and development[37–39]. In the GDP-bound form, RAS is inactive. A crucial event in RAS activation is the exchange of GDP for GTP. The conversion of RAS-GDP to the signalling-competent RAS-GTP is catalysed by GDP/GTP-exchange factors. In T and B cells, the RAS guanine-nucleotide releasing proteins (RAS-GRPs) have an important role in antigen-receptor-mediated RAS activation, whereas other receptors, such as the EGF receptor, mainly use the exchange factor son-of-sevenless (SOS)[40–42]. A recently determined crystal structure of the SOS–RAS complex provided a surprising insight into the regulation of SOS activity[43]. In this structure, SOS binds two RAS molecules, forming a ternary complex (FIG. 3b). One RAS molecule binds to the catalytic domain, whereas the second RAS molecule binds to one of the regulatory domains of SOS. This ternary complex structure implies that SOS is subject to positive feedback by its product RAS-GTP (FIG. 3b).

Positive-feedback loops other than direct enzyme–product feedback have also been found. Bruton's tyrosine kinase (BTK) is a component of a multimeric signalling complex (signalosome) that controls, among other events, the release of calcium in activated B cells[44]. After BCR ligation, the enzyme PI3K becomes active and generates the second messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3), which is a binding target for proteins with PH domains, including BTK (FIG. 4). Binding of the PH domain of BTK to PtdIns-3,4,5-P_3 not only localizes BTK to the cytoplasmic face of the plasma membrane, but also results in activation of the kinase domain. So, BTK requires PI3K for its activation, whereas PI3K requires the substrate PtdIns-4,5-bisphosphate (PtdIns-4,5-P_2) to produce PtdIns-3,4,5-P_3. The crucial PI3K substrate is produced by PtdIns-4-phosphate 5-kinases (PIP5Ks). It has now been found that the PH domain of BTK binds to PIP5K, and that this binding is part of a positive feedback that increases the PtdIns-4,5-P_2 concentration and, therefore, the synthesis of PtdIns-3,4,5-P_3 by PI3K[10,45]. The recruitment of the BTK–PIP5K complex to the membrane ensures that PI3K does not run out of its substrate and can rapidly increase the PtdIns-3,4,5-P_3-containing and BTK-binding membrane patch in activated lymphocytes. Furthermore, phospholipase C (PLC), another binding partner of the BTK-containing signalosome, also uses PIP5K-generated PtdIns-4,5-P_2 as a substrate to produce the second messenger inositol-1,4,5-trisphosphate (IP_3), which releases intracellular calcium stores. So, the PIP5K–BTK complex is not only involved in feedback loops, but also in forward signalling.

Another example of a more distant positive-feedback loop is the phosphorylation and activation of SYK by a downstream signalling element, the serine/threonine kinase extracellular signal-regulated kinase (ERK)[7] (FIG. 4). This feedback loop was detected by blockade of the ERK pathway by pharmacological inhibition of MEK, which resulted in decreased SYK activation in mast cells stimulated through Fcε receptor I (FcεRI). However, the position of the ERK phosphorylation site and the mechanism by which SYK activity is enhanced by this feedback have not been elucidated yet.

Negative-feedback loops

The duration of an amplified signal is restricted by negative-feedback loops that act as built-in brakes, which are automatically activated once a certain signal strength or duration has been reached. A negative-feedback loop is characterized by a signalling element that dampens the activity of its direct or indirect upstream activator through post-translational modification or the formation of new protein–protein interactions. The loss or mutation of signalling elements involved in negative-feedback loops might result in pathological hyperactivation of signalling pathways and diseases, such as tumour development or autoimmunity[11,12,46,47]. For example, hyper-responsive B cells and the secretion of autoantibodies are observed in mice with loss-of-function mutations in the LYN–CD22–SHP1 negative-feedback

loop[48–51]. At the cellular level, two different modes of negative-feedback loops can be distinguished: immediate negative feedback that occurs within seconds or minutes after signal perception and delayed negative feedback involving the *de novo* synthesis of a negative regulator.

Negative-feedback loops of the first category are often characterized by the phosphorylation of an upstream activator element through a downstream situated kinase. For example, BTK is negatively regulated in this way by protein kinase C-β (PKC-β). After BCR ligation, BTK becomes active and as part of the signalosome mediates the opening of intracellular calcium channels (FIG. 4). The released calcium then activates PKC enzymes such as PKC-β, which, among other targets, phosphorylates Ser180 in the TEC linker region of its upstream activator BTK. This phosphorylation stabilizes the autoinhibitory conformation of BTK and so counteracts its activity[52]. As indicated by this example, PKCs, as well as other serine/threonine kinases, are often involved in negative-feedback loops. Indeed, serine/threonine residues are often found close to tyrosine residues in the cytoplasmic tails of the EGF receptor and Ig-α, and it has been shown that these serine/threonine residues negatively regulate the activity of these receptors by still ill-defined mechanisms[53–55].

The importance of serine/threonine phosphorylation sites as targets for negative-feedback regulation is also highlighted by the finding that point mutations in these residues can transform adaptor proteins into potent oncoproteins[11,12]. The adaptor protein FGF receptor substrate 2α (FRS2α) is a key mediator of signalling through the FGF, nerve growth factor (NGF) and glial-cell-derived nerve growth factor (GDNF) receptors, which are also expressed by haematopoietic cells and are potent inducers of the ERK pathway[56–58]. FRS2α contains eight threonine residues, which are phosphorylated by ERK[12]. A mutant of FRS2α, with all eight threonines exchanged for valines, has increased tyrosine phosphorylation, enhanced association with growth factor receptor-bound protein 2 (GRB2) and confers anchorage-independent growth of fibroblasts in soft agar — a hallmark of malignant transformation. A second example is the adaptor protein GRB2-associated binding protein 2 (GAB2), which is tyrosine phosphorylated after antigen-receptor ligation and in cells expressing the BCR–ABL oncoprotein[59]. Tyrosine phosphorylated GAB2 is associated with the p85 regulatory subunit of PI3K and is involved in the activation of the ERK and PI3K/PKB pathways[11,59]. The downstream signalling element PKB can phosphorylate GAB2 at Ser159, and this event negatively regulates GAB2 tyrosine phosphorylation downstream of ERBB receptors in 293 T cells. A Ser159Ala mutant of GAB2, which is consequently uncoupled from this negative-feedback phosphorylation, has transforming properties[11].

Many examples of negative-feedback loops are also found in the classical mitogen-activated protein kinase (MAPK)/ERK pathway. The activity of the ERK pathway is tightly controlled and its dysregulation has been implicated in malignant transformation[60]. Although this pathway is engaged by numerous receptors, the activity of this pathway also has a decisive role in antigen-receptor-mediated development of B and T cells and in the acquisition of their effector functions[4–6,37,61–64]. Therefore, it is not surprising that several negative-regulatory loops impinge at almost every level of this cascade (FIG. 4). For example, ERK phosphorylates and dampens the activity of its upstream signalling components MEK1[65–68], RAF1 and BRAF[8,69–71], as well as the guanine-nucleotide exchange factor SOS[72–75]. Although the feedback phosphorylation of SOS and

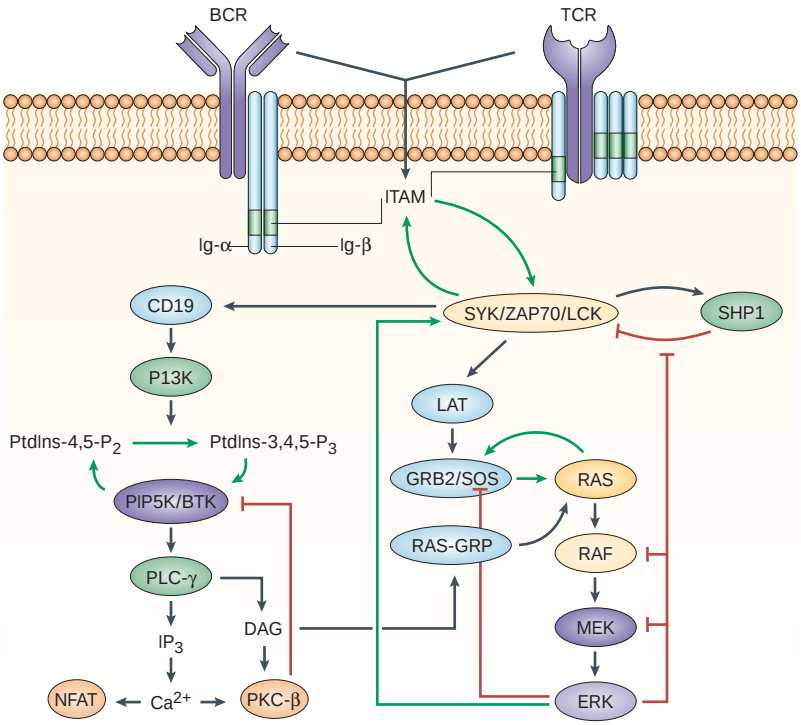

Figure 4 | **Examples of positive- and negative-feedback loops downstream of antigen receptors on T and B cells.** The ligation of antigen receptors (B-cell receptors, BCRs, or T-cell receptors,TCRs) results in activation of protein tyrosine kinase (PTK) of the SYK/ZAP70- and SRC-family kinases and the subsequent phosphorylation of adaptor proteins, such as linker for activation of T cells (LAT) in T and pre-B cells[103] or Bam32 in B cells (not shown)[104,105]. Ultimately, these events lead to increased levels of RAS-GTP through the recruitment of the GRB2–SOS complex to the BCR/TCR-proximal signalling complex through the SRC-homology 2 (SH2) domains of GRB2. Furthermore, marked levels of RAS-GTP are produced by diacylglycerol (DAG)-dependent membrane recruitment of RAS guanine-nucleotide releasing proteins (RAS-GRPs)[40–42]. In turn, RAS-GTP activates the serine/threonine kinases RAF1 and BRAF, which are co-expressed by T and B cells and are both involved in extracellular signal-regulated kinase (ERK) activation[71,106]. Many cytoplasmic ERK substrates lie upstream of ERK in this pathway and are therefore either positively regulated (for example, SYK, green arrow) or inhibited by ERK-mediated phosphorylation (red lines). Antigen-receptor-mediated activation of phosphatidylinositol 3-kinase (PI3K) can occur through the recruitment of the regulatory PI3K subunit p85 to tyrosine phosphorylated CD19. Activated PI3K converts PtdIns-4,5-bisphosphate (PtdIns-4,5-P_2) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3), which tethers signalling molecules such as Bruton's tyrosine kinase (BTK) to the membrane. The PtdIns-4,5-P_2-producing PtdIns-4-phosphate 5-kinase (PIP5K) associates with BTK and is brought to the membrane with BTK[10]. This event supplies the membrane-resident enzymes PI3K and phospholipase C-γ (PLC-γ) with their substrate (PtdIns-4,5-P_2) and results in signal amplification by positive feedback (green arrows). The increasing PLC-γ activity results in production of the secondary messengers DAG and inositol-1,4,5-trisphosphate (IP_3). DAG and calcium ions also synergize in the activation of protein kinase C-β (PKC-β), which inhibits BTK signalling by a negative-feedback loop (red line) through the phosphorylation of serine 180 (REF. 52). GRB2, growth factor receptor-bound protein 2; ITAM, immunoreceptor tyrosine-based activation motif; NFAT, nuclear factor of activated T cells; SHP1, SH2-domain-containing protein tyrosine phosphatase 1; SOS, son-of-sevenless.

RAF1 was first identified in non-lymphoid cells, it has also been observed in antigen-receptor-stimulated B cells[71,76], which is not surprising given the ubiquitous expression of these molecules. Indeed, the feedback phosphorylation of SOS, RAF1 and BRAF can be easily analysed by their electrophoretic mobility shift in whole lysates from BCR-stimulated B cells[8,71,76]. However, despite a decade of research on the feedback phosphorylation of SOS and RAF1, there is still a debate about the precise molecular mechanism by which this feedback affects their function. For example, after stimulation with insulin, platelet-derived growth factor (PDGF) or vascular endothelial growth factor (VEGF), SOS phosphorylation results in dissociation of the GRB2–SOS complex[73,77,78]. By contrast, phosphorylated SOS remains bound to GRB2 in phorbol-ester-stimulated T cells, but this phosphorylation prevents the binding of the GRB2–SOS complex to tyrosine phosphorylated proteins such as linker for activation of T cells (LAT)[79,80]. It is possible that in the latter case, a second negative-feedback loop directly prevents binding of the GRB2–SOS complex to receptor molecules. Nevertheless, these studies clearly show that RAS-GTP levels are limited by the ERK-mediated feedback phosphorylation of SOS, which is illustrated by the fact that the MEK inhibitor PD98059 prolongs the production of RAS-GTP[73,81]. This observation is also of potential relevance for the therapy of RAS-GTP-dependent tumours, as it shows that inhibition of the RAS effector MEK could generate sustained levels of RAS-GTP, which in turn might cause the undesirable activation of other transforming RAS effectors, such as the PI3K/PKB or RAL-GEF pathways[36].

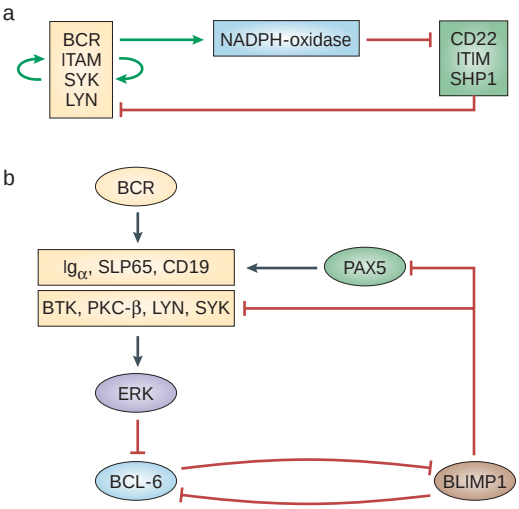

Figure 5 | **Double-negative feedback loops. a** | Model of B-cell receptor (BCR)-signal amplification by a double-negative feedback loop. Under basal conditions, the activity of SYK is tightly controlled by the protein tyrosine phosphatase SHP1, which binds to the phosphorylated immunoreceptor tyrosine-based inhibitory motif (ITIM) tyrosines of the co-receptor CD22 and efficiently dephosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) of Ig-α, as well as BCR-proximal signalling proteins such as SH2-domain-containing leukocyte protein of 65 kDa (SLP65) and phospholipase C-γ (PLC-γ)[107–111]. BCR ligation and translocation can result in the local production of oxygen radicals, which inhibit SHP1 through the reversible oxidation of a cysteine residue in the catalytic site of SHP1 (REF. 85). In the absence of SHP1 activity, the positive SYK/ITAM feedback loop causes signal amplification and diversification. **b** | Control of B-cell development by a bi-stable double-negative feedback loop. B-cell development is controlled by a bi-stable system consisting of B-cell lymphoma 6 (BCL-6) and B-cell-induced maturation protein 1 (BLIMP1)[90,91]. Expression of some of the key elements involved in BCR signalling, such as SLP65, Ig-α and CD19, depends on the transcription factor paired box 5 (PAX5), which is turned off by BLIMP1 during plasma-cell differentiation. BLIMP1 also represses the expression of other BCR-signalling elements, including Bruton's tyrosine kinase (BTK), protein kinase C-β (PKC-β), LYN and SYK[92]. So, expression of BCR-signalling elements in mature and germinal-centre B cells is ensured by expression of BCL-6, which represses the expression of BLIMP1. However, strong BCR-derived signals, which trigger the terminal differentiation of germinal-centre B cells into plasma cells, lead to phosphorylation of BCL-6 by activated extracellular signal-regulated kinase (ERK) and proteasomal degradation[93,94], and subsequent release of the transcriptional repression on the *BLIMP1* gene. In turn, BLIMP1 represses the expression of its own repressor BCL-6 and terminates the expression of BCR-signalling elements. SHP1, SRC-homology-2-domain-containing protein tyrosine phosphatase 1.

In addition to the immediate negative-feedback loops, several examples for late, transcription-dependent, negative-feedback loops have been discovered in cells of the immune system. For example, transcription of the suppressor of cytokine signalling (SOCS) family genes is activated by signalling through the Janus-family kinase/signal transducer and activator of transcription (JAK/STAT) pathway[82]. Once produced, the SOCS PROTEINS bind to cytokine receptors, where they inhibit JAK activation and STAT phosphorylation, thereby constituting negative-feedback regulation. Interestingly, hypermethylation and consequently loss of expression of the *SOCS1* gene is frequently found in myelomas, which is in accordance with the importance of an active JAK/STAT pathway in myelomagenesis[47]. Another example of a delayed negative-feedback loop is that which controls activation of the transcription factor nuclear factor-κB (NF-κB), which induces the *de novo* synthesis of its own repressor, inhibitor of NF-κBα (IκBα). Recent work by Hoffmann and colleagues[83] showed that the interplay between NF-κB activation and *de novo* synthesis of IκBα results in oscillations of nuclear NF-κB. It should be noted that oscillatory systems are hallmarks of several negative-feedback loops[14].

Double-negative feedback loops and bi-stability

As described in a recent review, the biochemical reactions between signalling molecules are in principle reversible; however, signal transduction often leads to irreversible processes and all-or-nothing responses[14]. This signalling behaviour characterizes double-negative feedback loops or bi-stable signalling systems. Lineage decisions and differentiation pathways of haematopoietic cells are regulated by double-negative feedback loops. According to simple signalling logic, the outcome

JAK/STAT PATHWAY
The Janus-family kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is activated by numerous cytokines and growth factors. JAKs associate with cytokine receptors and become activated after ligand binding. Activated JAKs tyrosine phosphorylate the receptor and recruit STATs, which are also phosphorylated by activated JAKs. Phosphorylated STATs then dimerize and translocate to the nucleus, where they control gene transcription.

SOCS PROTEINS
The suppressors of cytokine signalling (SOCS) are a family of intracellular proteins that modulate the activity of cytokine receptors and receptor tyrosine kinases. SOCS proteins seem to regulate signal transduction by either direct inhibitory interactions with cytokine receptors and Janus-family kinases or by targeting associated proteins for degradation.

of the inhibition of an inhibitor is positive, resulting in signal amplification and prolongation of signalling long after the trigger stimulus is removed.

If double-negative feedback loops are combined with positive-feedback loops, they constitute a signalling system that displays a switch-like behaviour after activation. A good example of this is the initiation and amplification of intracellular signals from the BCR. The ITAM–SYK positive-feedback loop at the BCR is under strict negative regulation by protein tyrosine phosphatases such as SHP1 (REF. 9). This phosphatase binds to ITIM-containing co-receptor molecules, including CD22, CD72 and paired immunoglobulin-like receptor-B (PIR-B), that are located close to zthe BCR[84]. Due to its high turn-over rate, an active phosphatase can remove the phosphate groups from the tyrosine residue of an ITAM 10–100 times more efficiently than SYK can add them. Therefore, SHP1 efficiently inhibits ITAM–SYK-mediated signal amplification. However, the activity of SHP1 itself can be inhibited by oxidants such as H_2O_2 that oxidize a crucial cysteine residue in the catalytic site of SHP1 (FIG. 2b). B-cell activation is accompanied by an increase in H_2O_2 production and it is feasible, albeit not yet proven, that after its ligation, the BCR becomes co-localized with H_2O_2-producing oxidases. Under these circumstances, SHP1 loses its phosphatase activity and can no longer inhibit the positive ITAM–SYK feedback loop[85]. This inhibition of an inhibitor allows the rapid amplification of BCR signalling after antigen recognition (FIG. 5a).

Double-negative feedback loops also operate in T-cell receptor (TCR) signalling. In stimulated T cells, SHP1 is phosphorylated at Tyr564 (pSHP1) by the SRC-family kinase LCK. SHP1 phosphorylation provides a docking site for the SH2 domain of LCK and the formation of a LCK–pSHP1 complex, which terminates LCK-dependent signalling events such as activation of the ERK pathway. After strong TCR ligation, however, the ERK pathway becomes rapidly and more strongly activated, leading to the feedback phosphorylation of LCK at Ser59 by activated ERK[86–88]. Interestingly, phosphorylated Ser59 blocks formation of the LCK–pSHP1 complex and, therefore, LCK-dependent signalling events can be sufficiently sustained to trigger the production of autocrine growth factors and proliferation[89]. So, TCR signals are also amplified by a double-negative feedback, involving inhibition of the inhibitory LCK–SHP1 complex by an ERK-mediated negative feedback of LCK (FIG. 4). As ERK signalling influences the CD4/CD8 lineage decision of developing thymocytes[4–6], it would be interesting to analyse to what extent this double-negative feedback loop influences this developmental checkpoint.

Another example of a bi-stable signalling circuit is the regulation of the transcription factors B-cell lymphoma-6 (BCL-6) and B-cell-induced maturation protein 1 (BLIMP1) that together control the terminal differentiation of B cells[90,91]. The expression of BCL-6 is suppressed by BLIMP1, and conversely, BLIMP1 suppresses the expression of BCL-6 (FIG. 5b). In addition, BCL-6 supports the expression of genes involved in BCR signalling (for example, BTK, PKC-β, LYN, SYK, CD45 and CD21), whereas BLIMP1 represses them[92]. Furthermore, BLIMP1 also turns off the transcription factor paired box gene 5 (PAX5), which is required for the expression of BCR-signalling elements, including SH2-domain-containing leukocyte protein of 65 kDa (SLP65, also known as B-cell linker, BLNK), Ig-α and CD19. In this bi-stable system, BCL-6 maintains the identity of germinal-centre B cells, whereas BLIMP1 promotes plasma-cell differentiation. If BCR ligation, however, leads to robust ERK activation, the activated ERK will phosphorylate BCL-6, which promotes its degradation by the proteasome[93,94]. In the absence of BCL-6, transcription of the *BLIMP1* gene is initiated and the resulting BLIMP1 protein represses the further production of BCL-6 and that of the BCR-signalling elements mentioned above. So, strong BCR signals remove BCL-6 from the bi-stable BCL-6–BLIMP1 circuit and initiate an irreversible switch in the developmental programme, which drives the terminal differentiation of B cells into plasma cells[95].

Concluding remarks

Feedback circuits have an essential role in the regulation of intracellular signalling pathways, and it is probable that each intracellular signalling molecule is part of, and connected to, several such loops. Indeed, by reviewing recent work on BTK, SOS and LCK, we have illustrated that these molecules are embedded in a signalling network consisting of both positive- and negative-feedback loops. Yet, at present, only a few of these feedback loops have been discovered, and we are far from understanding the complex spatial and temporal interplay between these loops within signalling systems. One method that enables the discovery of signalling loops is the use of specific inhibitors for a given signalling molecule. If the inhibition of a downstream signalling element results in a change of activity (positive or negative) of an upstream signalling molecule, this can be taken as evidence for feedback regulation. For example, pharmacological inhibition of MEK1 enhances the activity of its upstream activator RAF1, supporting the model that RAF1 is subject to a MEK/ERK-dependent negative-feedback loop[69]. An alternative approach is the reconstruction of a signalling pathway using a collection of wild-type and mutant forms of the relevant signalling molecules[9].

It will be important to learn more about the feedback regulation of signalling pathways to increase our understanding of signalling in normal cells, as well as in diseases such as autoimmunity and tumour development, which are often caused by the dysregulation of intracellular signalling systems. A better understanding of the interplay between signalling loops in cells might make it possible to develop a combination of drugs that target crucial signalling feedback loops causing the altered or aberrant signalling behaviour of diseased cells. Finally, if more knowledge about the kinetic and quantitative aspects of these signalling loops becomes available, it will be possible to make more precise mathematical models that predict the signalling behaviour of a cell.

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Competing interests statement

The authors declare that they have no competing financial interests.

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