Molecular mechanisms of CD4^{+} T-cell anergy

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Abstract | Directing both innate and adaptive immune responses against foreign pathogens with correct timing, location and specificity is a fundamental objective for the immune system. Full activation of CD4^{+} T cells requires the binding of peptide–MHC complexes coupled with accessory signals provided by the antigen-presenting cell. However, aberrant activation of the T-cell receptor alone in mature T cells can produce a long-lived state of functional unresponsiveness, known as anergy. Recent studies probing both immune signalling pathways and the ubiquitin–proteasome system have helped to refine and elaborate current models for the molecular mechanisms underlying T-cell anergy. Controlling anergy induction and maintenance will be a key component in the future to mitigate unwanted T-cell activation that leads to autoimmune disease.

Central tolerance
The lack of self-responsiveness that occurs during lymphocyte development in the central lymphoid organs. B-cell progenitors in the bone marrow and T-cell progenitors in the thymus that strongly recognize self antigen either undergo further rearrangement of antigen-receptor genes to avoid reactivity to self or face deletion by apoptosis.

'The highest result of education is tolerance.'
Helen Keller

In the complex matrix of an immune response, tolerance to self allows the identification and elimination of non-self pathogens while simultaneously minimizing bystander damage to the host. To help accomplish this immense task, the immune system has a series of control elements that govern the T-cell response, beginning with the differentiation of early T-cell progenitors in the thymus through to the responses of mature T cells in the peripheral immune compartments. This 'tolerance framework' has evolved to encompass an efficient two-tiered system that operates both during thymic development and peripheral homeostasis. At the first level, thymic-regulated central tolerance removes developing thymocytes that strongly recognize self proteins from the immature T-cell population, while in the periphery a second level of control (known as peripheral tolerance) regulates the mature T-cell compartment against unwanted responses to self[1–3].

During the termination of an immune response, a small number of antigen-specific T cells differentiate towards a memory phenotype and provide long-term protection by enhancing both their lifespan and responsiveness to re-encounter with the same antigen. However, studies in the 1980s, using antigen-specific T-cell clones *in vitro* as a surrogate for memory responses, identified a new phenomenon wherein mature T cells could be instead rendered unresponsive to recall stimulation by encounter with antigen in the absence of co-stimulation[4]. This cell-intrinsic state of unresponsiveness, subsequently termed clonal T-cell anergy, was shown to be long-lived, associated with defects in cell-cycle progression and some effector function, and reversible with strong stimuli.

Due to the many different experimental conditions and model systems used, it has been difficult to build a cohesive mechanistic model of clonal T-cell anergy. Thus, over time, the term has expanded to include a range of cellular conditions that collectively share broad phenotypes such as diminished proliferation and cytokine production. Because a multitude of input signals are required to coordinate complex processes such as cell division, there are many opportunities for perturbations to produce effects that resemble clonal T-cell anergy. Although essential molecular components of T-cell signalling were an initial focus of investigation, recent data have demonstrated that E3 ubiquitin ligases of the ubiquitin–proteasome system are now also an essential component of the T-cell anergy phenotype.

In this Review, we first discuss the early work that contributed to the understanding of clonal T-cell anergy, as well as new data that have further refined these models. We then highlight the manner in which E3 ubiquitin ligases modulate both previously known and unknown pathways that control T-cell anergy induction. Ultimately, the importance for understanding and manipulating T-cell anergy is appealing to prevent or attenuate unwanted T-cell responses *in vivo*, most notably those associated with autoimmune diseases.

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doi:10.1038/nri2131
Published online 6 July 2007

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Early insights into clonal T-cell anergy

An extensive examination of the cellular and molecular events associated with both clonal T-cell anergy and the related *in vivo* adaptive tolerance has been undertaken previously[5]. Clonal T-cell anergy was initially characterized in $CD4^+$ T helper 1 (T_H1)-cell clones by stimulation through the T-cell receptor (TCR) without co-stimulation through CD28 (REFS 6–8). The model systems used for these studies included stimulation of T cells with chemically crosslinked antigen-presenting cells plus peptide, purified MHC class II molecules displaying the relevant peptide antigen in a planar lipid bilayer, or plate-bound CD3-specific antibody only. These heterogeneous systems provided a general framework to define clonal T-cell anergy as a long-lived state that is distinct from apoptosis induction and characterized by reduced cellular proliferation and cytokine production upon restimulation[9]. Specifically, anergized T-cell clones produced negligible amounts of interleukin-2 (IL-2), which is crucial for clonal expansion following T-cell activation. Interestingly, clonal T-cell anergy was found not to be a terminal fate, as the addition of exogenous IL-2 during restimulation could reverse the phenotype[10]. These characteristics are not limited to T cells, as anergic B cells also demonstrate some of the hallmark features, such as reduced proliferation and effector function[105].

The reduction in IL-2 production by restimulated anergic T cells was found to reside at the level of *Il2* transcription. In anergic T cells, DNA binding of the activator protein 1 (AP1) heterodimer, comprised of the transcription factors FOS and JUN, was reduced in reporter assays, and subsequent studies of signalling pathways in anergic T cells found defects in the activation of the immediate upstream molecules of the mitogen-activated protein kinase (MAPK) families[11–14]. Importantly, however, anergic T cells had no defects in the activation and nuclear translocation of nuclear factor of activated T cells (NFAT), which is activated following calcium flux, principally downstream of TCR crosslinking[15]. This observation correlates well with previous data showing that the induction of T-cell anergy could be blocked by the addition of cyclosporin A, an inhibitor of the immediate upstream activator of NFAT, calcineurin[8]. The fact that the T cells used for these studies were established from T_H1-cell lines that had been previously stimulated is important, because it is difficult to induce anergy in naive T cells *in vitro*, with plate-bound CD3-specific antibody being the only robust system reported[16,17]. Activation of the MAPK pathway that leads to formation of the AP1 complex is downstream of the small GTPase RAS, which is fully activated after co-stimulation through CD28 (REF. 18). In anergic T_H1-cell clones, the lack of activation of the MAPKs extracellular-signal-regulated kinase 1 (ERK1) and/or ERK2 was found to result from a defect in the GTP loading of RAS, even though its canonical guanine-nucleotide-exchange factor (GEF) son of sevenless homologue (SOS) was activated normally[12]. These data point to a block in proximal T-cell signalling at the level of RAS activation in anergic T cells, but this model has been difficult to reconcile mechanistically owing to the functional machinery that leads to RAS GTP loading (FIG. 1). One possible explanation for the block in RAS activation could be the expression or upregulation of proteins specific to the anergic state that are normally inactive or absent following full activation of T cells. This hypothesis is supported by the observations that the presence of the protein-synthesis inhibitor cycloheximide prevents T-cell anergy induction *in vitro* and that anergy can be transferred after heterokaryon formation[7,19].

A key distinction must be drawn here between *in vitro* clonal T-cell anergy and the T-cell unresponsiveness that is seen *in vivo*, and which is known as adaptive tolerance[5]. The phenomenon of adaptive tolerance was first described *in vivo* following the transfer of T cells bearing TCRs that bind the mouse mammary tumour virus superantigen Mtv-7 into mice with endogenous expression of this retroviral superantigen. Adaptive tolerance was also later shown to be induced in TCR $V\beta8^+$ T cells following the injection of the T-cell superantigen staphylococcal enterotoxin B (SEB)[20–22]. Since these early studies, the use of TCR-transgenic T cells transferred to recipient mice containing injected soluble antigen has helped to delineate key differences between adaptive tolerance and clonal anergy of T cells *in vitro*[23]. In this *in vivo* model, antigen challenge of TCR-transgenic mice results in a large amount of T-cell death but a small population of anergic T cells survives. These remaining T cells have reduced proliferation and do not produce IL-2, characteristics which resemble those of the clonal T-cell anergy state *in vitro*, but they also show decreased production of almost all cytokines and cannot be rescued by exogenous IL-2 (REFS 24, 25). A second difference between these two states is that *in vitro* clonal T-cell anergy is most often induced with previously activated T cells, whereas adaptive tolerance can be induced with naive T cells. These studies also revealed that *in-vivo*-anergized T cells slowly recovered functional capability over time owing to a loss of the injected antigen, which indicates that adaptive tolerance requires the persistence of antigen in the periphery[23,25,26]. By contrast, *in vitro* clonal T-cell anergy does not require persistent antigen. Finally, there is evidence suggesting that cytotoxic T-lymphocyte antigen 4 (CTLA4) could have a role in adaptive tolerance, but it is dispensable for clonal T-cell anergy[27,28].

Recent insights into clonal T-cell anergy

RASGRP1 and DAG. Although the prevailing hypothesis for the induction of clonal T-cell anergy proposes a block in RAS activation due to failed GTP loading and implies elevated levels of a GTPase-activating protein (GAP), no evidence has been shown to directly support this model. However, two groups reported that the defect in RAS activation and thereby downstream MAPK activation could be overcome by addition of the diacylglycerol (DAG) analogue phorbol 12-myristate 13-acetate (PMA) to cultures of T cells during restimulation[12,13]. It has long been known that there is a second pathway of RAS activation in T cells, which is independent of SOS and which is activated by the protein kinase C (PKC) activator PMA[29,30]. The GEF RAS guanyl-releasing

Peripheral tolerance
The lack of self-responsiveness of mature lymphocytes in the periphery to specific antigens. These mechanisms can control potentially self-reactive lymphocytes that have escaped central tolerance or prevent immune responses to specialized self proteins that were not present during establishment of central tolerance. Peripheral tolerance is associated with suppression of self-reactive antibody production by B cells and inhibition of self-reactive effector cells, such as cytotoxic T lymphocytes.

E3 ubiquitin ligase
An enzyme that attaches ubiquitin to substrate proteins. Single subunit E3 ubiquitin ligases contain both the substrate-binding domain(s) and E2-tranferase recruitment machinery in the same polypeptide chain, whereas multisubunit E3 ubiquitin ligases divide these functions between individual protein components. E3 ubiquitin ligases are further classified on the basis of their E2-transferase recruitment domains: HECT-type, RING-finger-type and U-box-type.

Adaptive tolerance
Also known as *in vivo* anergy, this phenomenon results from the challenge of T cells in the periphery by either superantigen or specific peptides that results in T-cell activation in the absence of upregulated co-stimulation on antigen-presenting cells. This state differs from *in vitro* clonal T-cell anergy in many ways, most notably in its requirement for persistent antigen presentation to maintain the anergic state.

Activator protein 1
(AP1). A transcription factor heterodimer of FOS and JUN that is formed following T-cell activation and is critically important for inducing the transcription of interleukin-2.

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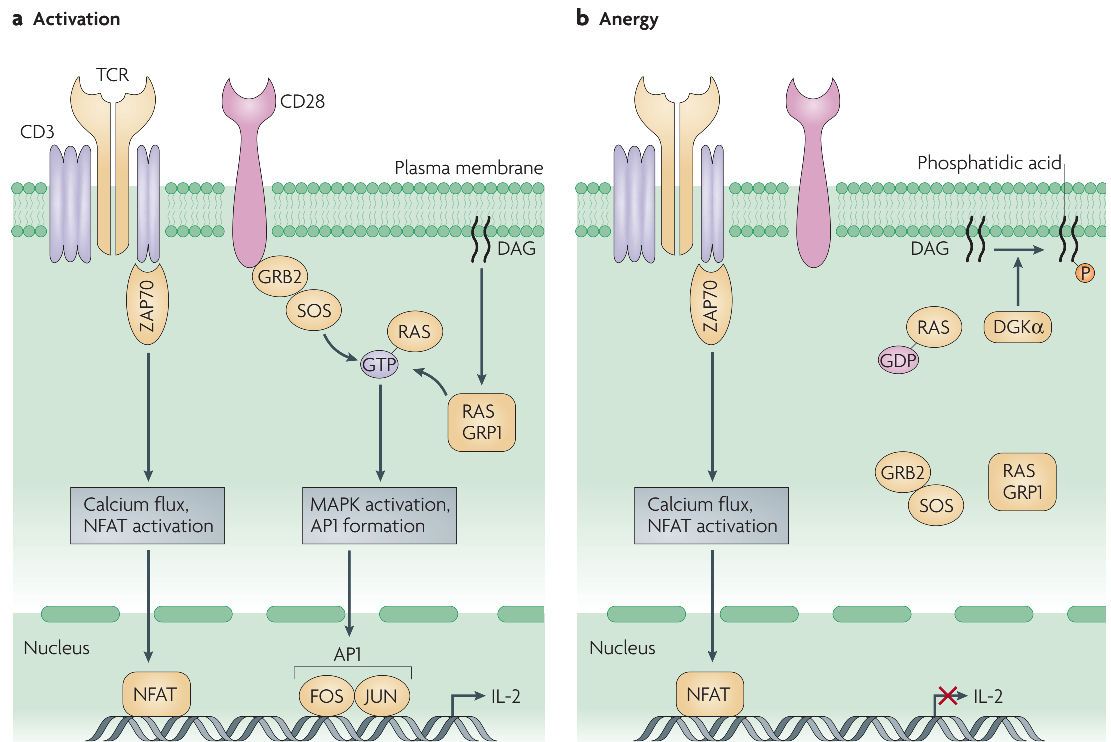

Figure 1 | **A comparison between signalling pathways in fully stimulated and in anergized T cells. a** | The initial stages (0–16 hours) of T-cell activation require input from both the T-cell receptor (TCR) and CD28 pathways, which converge to induce interleukin-2 (IL-2) production. **b** | Anergy induction requires the activation of calcium flux and nuclear factor of activated T cells (NFAT), but there are defects at various points in the MAPK (mitogen-activated protein kinase) pathway downstream of co-stimulation. Initial reports demonstrated that the small GTPase RAS was defective in GTP loading, and recent work has shown this could result from hyperconversion of the second messenger lipid diacylglycerol (DAG) to phosphatidic acid and failed recruitment of the guanine-nucleotide-exchange factor (GEF) RAS guanyl-releasing protein 1 (RASGRP1). Combined with the lack of recruitment to CD28 of another GEF, SOS (son of sevenless homologue), these effects prevent activation of the MAPK pathway for the formation of the activator protein 1 (AP1) complex in the nucleus and IL-2 production 16–24 hours following T-cell activation. DGKα, diacylglycerol kinase α; GRB2, growth-factor-receptor-bound protein 2; ZAP70, ζ-chain-associated protein kinase of 70 kDa.

Cyclosporin A
An immunosuppressive drug that inhibits calcineurin, a Ca^{2+}-dependent serine/threonine phosphatase necessary for the nuclear translocation of the transcription factor NFAT (nuclear factor of activated T cells).

Guanine-nucleotide-exchange factor
(GEF). A protein that stimulates the exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) on small GTPases, resulting in activation of the GTPase.

Heterokaryon
Any cell with more than one nucleus and the nuclei of which are not all of the same genetic constitution. The term heterokaryon can also refer to a tissue composed of such cells.

Superantigen
A microbial protein that activates all T cells expressing a particular set of T-cell receptor (TCR) Vβ chains by crosslinking the TCR to a particular MHC regardless of the peptide presented.

Immunological synapse
A region that can form between two cells of the immune system in close contact. The immunological synapse originally referred to the interaction between a T cell and an antigen-presenting cell. It involves adhesion molecules, as well as antigen receptors and cytokine receptors.

protein 1 (RASGRP1) was subsequently found to link DAG to the activation of RAS in T cells. RASGRP1 is recruited to the Golgi membrane by increases in the levels of DAG and intracellular calcium following T-cell activation[31,32]. At the Golgi membrane, RASGRP1 promotes the exchange of GDP for GTP on RAS, leading to subsequent activation of the downstream MAPK pathways[33,34]. Defects in this RASGRP1-mediated pathway of RAS activation might be involved in the induction of T-cell anergy, which is consistent with the observations that RASGRP1-deficient thymocytes are defective in signalling, proliferation and maturation[35].

Two recent studies have found that the amount of DAG limits the TCR-signalling ability of anergic T cells, as decreased DAG levels are responsible for reducing the recruitment of RASGRP1, which is needed for the activation of RAS[36,37]. DAG signalling is quenched by its conversion through phosphorylation to phosphatidic acid by diacylglycerol kinases (DGKs). Because it was previously shown that DGKα was differentially upregulated in anergic T cells and that a related family member DGKζ could antagonize TCR signalling, modulation of DAG by DGKα following T-cell activation could facilitate anergy induction[38–40]. DGKα overexpression was shown to selectively inhibit AP1 activation but did not affect the calcium flux upon T-cell activation. In addition, peripheral T cells from DGKα-deficient mice, compared with cells from wild-type mice, had hyperactive DAG-dependent signalling downstream of the TCR and were resistant to anergy induction[36]. Conversely, RASGRP1 recruitment to the immunological synapse was reduced in anergic T cells, a process that could be recapitulated by DGKα overexpression[37]. Importantly, these authors made the observation that expression of constitutively active RAS in anergic T_H1-cell clones restored IL-2 production and MAPK activity[41]. Together, these results support a model of clonal T-cell anergy in which defective RAS activation is an active process that can result from differentially expressed inhibitors of DAG following T-cell activation (FIG. 1).

Early growth response genes. An immediate target of NFAT-mediated transcription following calcium flux in T cells is the early growth response (EGR) family of transcription factors. Of the four members of this family, EGR1 is the most extensively studied, and its

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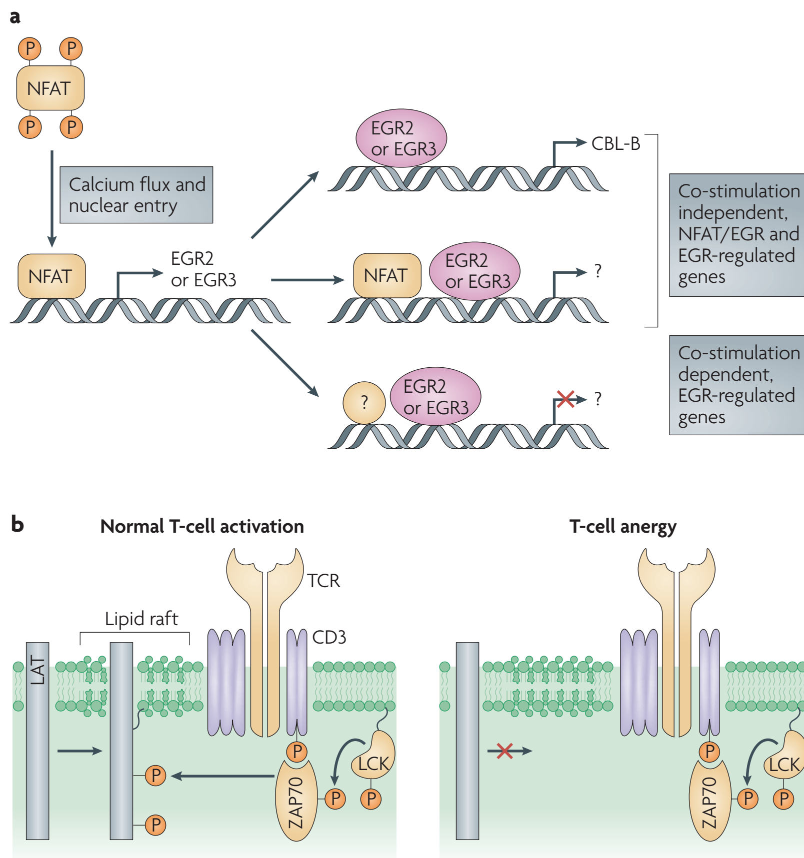

Figure 2 | **Two recent additions to the mechanism of anergy induction.** **a** | Following calcium flux in T cells, activated NFAT (nuclear factor of activation T cells) traffics to the nucleus and upregulates the transcription of several genes, including the genes encoding the transcription factors early growth response 2 (EGR2) and EGR3. As members of the EGR family are normally required for proper T-cell activation, they could synergize with NFAT and other upregulated transcription factors. However, during anergy induction, the possible exclusion of these other associated transcription factors, owing, for example, to a lack of complete co-stimulation, could lead to EGR2 and EGR3 instead giving rise to an anergy signature. **b** | Linker for activation of T cells (LAT) is a transmembrane adaptor protein that is phosphorylated and palmitoylated following T-cell activation. LAT provides a surface for the interaction of signalling components that are responsible for both the propagation and dissemination of the T-cell signal from the plamsa membrane to the cellular interior. Following the induction of T-cell anergy both *in vitro* and *in vivo*, LAT is hypopalmitoylated and thus terminates T-cell signalling at elements proximal to the plasma membrane. CBL-B, Casitas B-lineage lymphoma B; DAG, diacylglycerol; TCR, T-cell receptor; ZAP70, ζ-chain-associated protein kinase of 70 kDa.

Small interfering RNA (siRNA). Synthetic RNA molecules of 19–23 nucleotides that are used to 'knockdown' (that is, silence the expression of) a specific gene. This is known as RNA interference (RNAi) and is mediated by the sequence-specific degradation of mRNA.

expression is rapidly upregulated following various stimulatory events in a range of cells from neuronal cells to leukocytes[42]. Microarray analysis of the mouse CD4$^+$ T_H1-cell clone A.E7 either anergized with CD3-specific antibody alone or fully stimulated with both CD3-specific antibody and CD28-specific antibody indicated that high levels of expression of EGR2, but not EGR1, persisted for 2 or 5 days in anergized T cells[43]. Treatment of T cells with *Egr2*-targeted small interfering RNA (siRNA) before antibody stimulation indicated that EGR2 expression was required for the induction of anergy. In a subsequent study also using CD3-specific-antibody-treated A.E7 T-cell clones, screening analysis confirmed the rapid upregulation of expression of EGR2 and EGR3 in anergized cells both *in vitro* and *in vivo*, and indicated that their expression was dependent on the activity of calcineurin and PKC[44]. Importantly, the transduction of T cells with *Egr2* or *Egr3* decreased *Il2* transcription, and EGR3-deficient T cells were resistant to anergy induction by *in vivo* peptide administration. Together these results indicate that these EGR proteins have a role in conferring the anergic state. As *Egr2* and *Egr3* are both immediate targets of NFAT-mediated transcription in T cells, transcriptional complexes of NFAT and EGR proteins or the EGR proteins alone could be responsible for induction of the anergic programme (FIG. 2).

LAT. Linker for activation of T cells (LAT) functions as a transmembrane adaptor molecule that is recruited to the immunological synapse following TCR stimulation owing to its rapid phosphorylation and palmitoylation. Lacking intrinsic enzymatic activity, LAT instead is phosphorylated on five tyrosine residues, which leads to the recruitment of the signalling molecules GRB2 (growth-factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein), SLP76 (SH2-domain-containing leukocyte protein of 76 kDa) and PLCγ1 (phospholipase Cγ1) to propagate and disseminate intracellular signalling pathways[45]. A recent study has shown that peripheral CD4$^+$ T cells that are anergized both *in vitro* and *in vivo* have signalling defects downstream of LAT[46]. Defects in LAT palmitoylation were shown to prevent the recruitment of LAT to the immunological synapse and detergent-resistant microdomains in the membranes of anergized cells (FIG. 2). As this phenotype did not change total protein levels of LAT, these data indicate that clonal anergy can result from signalling defects owing to aberrant localization of key intracellular components. In addition, the protein(s) responsible for LAT palmitoylation is not only a key component of T-cell activation but is also itself susceptible to control by the anergy programme.

The ubiquitylation pathway and T-cell anergy

Although the regulation of cell differentiation, function and homeostasis lies principally at the level of gene transcription and mRNA stability, recent evidence suggests important roles for the ubiquitin–proteasome pathway in governing these cellular events. First discovered in 1975 from thymus extracts and later named for its omnipresent expression in all tissues, the 76-amino-acid globular ubiquitin molecule serves as the key informational component that ultimately encodes the fate of the protein to which it is attached[47,48]. Ubiquitin serves as the core molecule for the ubiquitylation pathway, with other components functioning in ubiquitin attachment (the coordinated activities of E1, E2 and E3 ubiquitin enzymes), ubiquitin removal (deubiquitylating enzymes) and the interpretation of ubiquitylated proteins (ubiquitin-binding proteins) (BOX 1). In most cases, ubiquitin attachment targets a protein for destruction by the 26S proteasome, but other fates, such as altered subcellular trafficking, conformational changes, activation and even protein stabilization, can occur[49].

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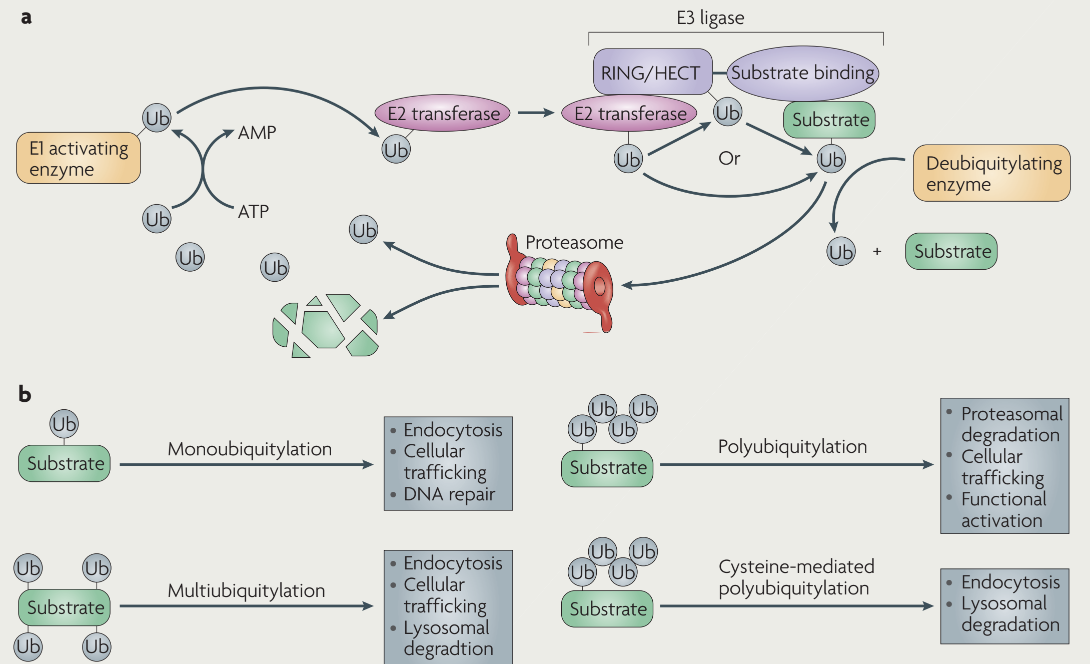
Detergent-resistant microdomains
(DRMs). Cell-membrane extracts that are enriched in cholesterol, phospholipids and sphingolipids, which are liquid ordered yet insoluble in non-ionic detergents. They are often referred to as lipid rafts, which provide ordered structure to the lipid bilayer and have the ability to include or exclude specific signalling molecules and complexes.

Ubiquitin-binding proteins
Proteins that contain one of the structurally diverse domains that can directly bind (generally with weak to moderate affinity) to ubiquitin attached to substrates. These domains include the ubiquitin-interacting motif (UIM), the ubiquitin associated (UBA) domain and the ubiquitin-conjugating enzyme variant (UEV) domain.

Box 1 | **Machinery of the ubiquitin–proteasome pathway**



The transfer of ubiquitin to a substrate protein (ubiquitylation) involves the serial transfer of ubiquitin across three protein elements of increasing specificity, to ensure attachment to the correct target in both time and space[102]. The carboxy terminus of the terminal glycine residue of ubiquitin is first converted in an ATP-dependent manner by an E1 ubiquitin-activating enzyme to a free thioester. This 'activated' ubiquitin is subsequently transferred to an E2 ubiquitin-conjugating enzyme, also commonly known as an E2 ubiquitin transferase. This E2 enzyme then binds to the recruitment domain of an E3 ubiquitin ligase (such as the RING (really interesting new gene) domain or the HECT (homologous to E6-AP carboxy terminus) domain), which in turn serves as a terminal adaptor by bringing together both the substrate (through its substrate-binding domain) and the activated ubiquitin. E1, E2 and some E3 ubiquitin enzymes carry activated ubiquitin in a thioester bond, but ubiquitin is most commonly added to the target protein via a covalent isopeptide bond between the carboxy group of its terminal glycine residue and the amino group of a lysine on the substrate protein. Isopeptide-bonded ubiquitin can be removed by deubiquitylating enzymes that can antagonize or even rearrange ubiquitin bonds to achieve specific outcomes[103] (see figure, part **a**).

Unlike other post-translational modifications that involve small chemical groups, such as phosphorylation, the amino-acid composition of ubiquitin allows for large flexibility in the construction of informational tags on target proteins[98]. Since ubiquitin itself contains seven lysine residues, an E3 ubiquitin ligase can continue to attach multiple ubiquitins together to form a polyubiquitin chain. Here, the ubiquitin lysine used for polyubiquitin chain synthesis, along with the absolute number of ubiquitin molecules added, encodes the ultimate fate of the substrate. A recent report also suggested the primary attachment of ubiquitin to non-lysine residues of cell-surface MHC class I molecules resulted in their removal from the cell surface[104] (see figure, part **b**).

Many reports have implicated an important role for the activity of E3 ubiquitin ligases in the regulation of T-cell activation, similar to what has been seen with negative regulation of T-cell activation by the E3 ubiquitin ligases SLIM (STAT-interacting LIM protein) and Deltex1, as well as with positive regulation of T-cell activation by the E3 ubiquitin ligase TRAC1 (T-cell RING (really interesting new gene) protein identified in activation screen 1; also known as RNF125)[50–52]. Another putative E3 ubiquitin ligase, ROQUIN, was found to control elements of co-stimulation, and accordingly, a single point mutation in ROQUIN identified in Sanroque (*san/san*) mice was shown to give rise to symptoms similar to those observed in the autoimmune disease systemic lupus erythematosus[53]. However, only a small cohort of E3 ubiquitin ligases — CBL-B (Casitas B-lineage lymphoma B), GRAIL (gene related to anergy in lymphocytes) and ITCH (itchy homologue E3 ubiquitin protein ligase; also known as AIP4) — have reproducibly been found to be associated with tolerant or anergic T cells[44,54,55] (FIG. 3).

Ionomycin-induced anergy and ubiquitin. As discussed earlier, one of the molecular hallmarks of the induction of T-cell anergy that is observed in most model systems is the normal translocation of NFAT to the nucleus in response to the calcium flux that results from TCR engagement. Therefore, if NFAT translocation to the nucleus is blocked by the addition of cyclosporin A to cell cultures, or if T cells lack NFAT1 expression, anergy induction is prevented[8,40]. On this basis, another robust model system for examining T-cell anergy was developed

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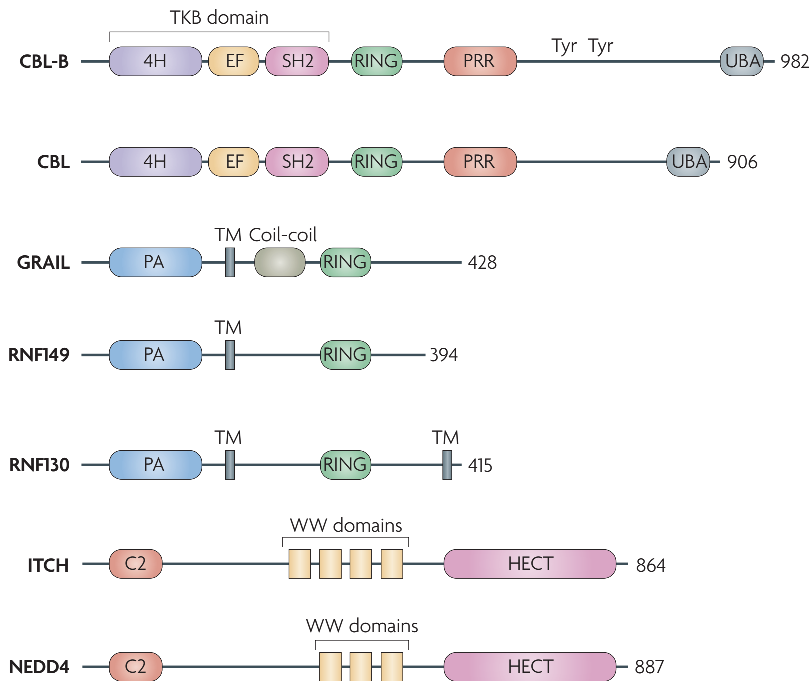

Figure 3 | **Structural domains of the anergy-associated E3 ubiquitin ligases.** There is little homology and few conserved domains between the CBL-B (Casitas B-lineage lymphoma B), GRAIL (gene related to anergy in lymphocytes) and ITCH (itchy homologue E3 ubiquitin protein ligase)/NEDD4 (neural precursor cell expressed, developmentally downregulated 4) protein families. Cytosolic CBL-B targets phosphorylated substrates mainly near the plasma membrane following T-cell activation, GRAIL is a transmembrane protein that is predominantly restricted to endosomes, and ITCH targets a wide variety of proteins, including cell-surface receptors, endomembrane-associated proteins and transcription factors. Therefore, the lack of homology suggests that these E3 ubiquitin ligases could target distinct substrates in various pathways and cellular compartments that are crucial for T-cell activation. The SRC homology (SH2) domain can bind phosphotyrosine residues; the RING (really interesting new gene)-finger domain recruits E2 ubiquitin transferases with activated ubiquitin; the proline-rich region (PRR) binds to SH3 domains; the ubiquitin associated (UBA) domain can bind to ubiquitin and ubiquitin-like proteins; the protease associated (PA) domain is speculated to be a protein–protein interaction domain; the coiled-coil region is a protein–protein interaction domain; C2 is a conserved region from protein kinase C that binds various lipids, calcium ions and other proteins; WW is a conserved dual tryptophan motif that binds proline-rich regions; homologous to E6-AP carboxy terminus (HECT) is a domain that recruits E2 ubiquitin transferases with activated ubiquitin and itself can bind ubiquitin via a thioester bond. 4H, four helix; EF, EF-hand-like; RNF, RING-finger protein; TM, transmembrane domain.

using the calcium ionophore ionomycin to propagate NFAT signals without the provision of co-stimulation. In this system, the presence of NFAT, but not AP1, in the nucleus was shown to trigger a gene-expression profile that is specific for anergy induction at the expense of T-cell activation[40]. Ionomycin-induced T-cell anergy differs slightly from other previously described models of clonal anergy induction, such as CD3-specific antibody treatment, because it involves impaired calcium mobilization following TCR restimulation (see later)[54]. However, some elements of *in vitro* T-cell clonal anergy are common to both ionomycin-induced and *in vitro* clonal anergy, suggesting commonality between these model systems. Interestingly, the analysis of ionomycin-treated T_H1-cell clones showed that expression of the E3 ubiquitin ligases CBL-B, GRAIL and ITCH was upregulated compared with controls, and this upregulation was sensitive to treatment with cyclosporin A[54,44,56]. How then does the expression of these three E3 ubiquitin ligases, which have little overlap in sequence homology or known protein substrates, lead to the induction of T-cell anergy?

Ionomycin
A calcium ionophore from *Streptomyces conglobatus* that induces the release of intracellular calcium stores.

CBL-B. CBL-B is a protein of 982 amino acids that was originally cloned from a breast cancer cell line and that contains high sequence homology to CBL[57]. Like CBL, CBL-B is inducibly phosphorylated by both the SYK (spleen tyrosine kinase)- and SRC-family kinases after TCR activation, and it is found constitutively associated with GRB2 (REF. 58). Interestingly, *Cblb*$^{-/-}$ mice developed autoimmunity either spontaneously by 6 months of age or following immunization with myelin basic protein (MBP)[59,60]. Although thymocyte development was essentially normal, peripheral T cells from *Cblb*$^{-/-}$ mice were hyperproliferative and secreted higher amounts of IL-2 after stimulation than wild-type cells. Because the level of TCR stimulation that is required to elicit a normal T-cell response is decreased in CBL-B-deficient T cells, CBL-B might help to set the threshold for T-cell activation in the periphery. This hypothesis was supported by the observation that a lack of co-stimulation of T cells through CD28 did not change the phenotype of CBL-B-deficient T cells, which suggests that CBL-B uncouples proliferation and IL-2 production from CD28 signalling[59,60]. In addition, a decrease in CBL-B protein levels following co-stimulation implies that a competent CD28 signal is sufficient to license the T cell for activation and to remove CBL-B in the process[61,62].

The mechanism used by CBL-B to attenuate peripheral T-cell activation involves the regulation of RHO-family GEF VAV1, as *Cblb*$^{-/-}$ T cells show enhanced and prolonged activation of VAV1, even without co-stimulation through CD28. These data confirmed earlier findings indicating that CBL-B inhibited the VAV-mediated activation of JUN[63]. Indeed, in response to CD3-specific antibody alone, *Cblb*$^{-/-}$ T cells showed efficient clustering of their TCRs and lipid-raft reorganization, processes that normally depend on CD28 co-stimulation through VAV1 (REFS 64,65). In addition, mice deficient in both CBL-B and VAV1 had normal T-cell function, adding further support to CBL-B modulation of T-cell activation through VAV1 and actin reorganization. CBL-B was also found to bind and ubiquitylate the p85 subunit of phosphoinositide 3-kinase (PI3K), but rather than induce proteasomal degradation, CBL-B-mediated ubiquitylation inhibited PI3K from associating with CD28 and TCRζ following T-cell activation[66,67]. As the formation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) by PI3K regulates the GDP–GTP exchange activity of VAV1, CBL-B can indirectly prevent activation of the RHO-family GTPases, TCR clustering and actin reorganization downstream of TCR activation[68].

Based on previous studies of anergic T cells *in vitro*, the regulation of signalling emanating from CD28 makes CBL-B a prime candidate for determining

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whether a T cell gains effector function and undergoes clonal expansion or is diverted to a long-lived anergic state. Experiments using *Cblb*$^{-/-}$ mice showed that CBL-B deficiency prevented anergy induction both *in vitro* and *in vivo*, and that exposure of *Cblb*$^{-/-}$ mice to multiple tolerogenic stimuli *in vivo* causes lethality[69]. So, the expression of CBL-B is thought to be crucial for helping to impart T-cell tolerance following antigen exposure. Interestingly, because EGR2 and EGR3 have been shown to upregulate CBL-B expression, this might explain how calcium flux can result in the upregulation of expression of E3 ubiquitin ligases during T-cell activation[44]. Finally, it has also been suggested that CBL-B, together with the related E3 ubiquitin ligase CBL, promotes the formation of a tolerogenic signalsome following T-cell activation by recruiting endocytic machinery to activated TCRs. Removing these signalling receptors from the cell surface and into endosomes could fundamentally change the signalling pathway away from activation and towards anergy[70].

It is well known that T cells downregulate their TCR–CD3 complexes after stimulation in an ubiquitin-dependent manner, and this reduction in TCR signalling capacity was thought to help control peripheral autoreactive T cells[71–74]. A clue to how this regulation might be achieved was shown by studying *Cbl*$^{-/-}$ mice, as thymocytes from these mice had higher levels of cell-surface expression of TCR and CD3 compared with cells from wild-type mice[75]. Recently generated knock-in mice that have a point mutation in the RING-finger domain of CBL show similarly increased levels of CD3 and TCR expression, suggesting that CBL regulates cell-surface expression of TCR and CD3 through a ubiquitin-mediated process[76]. Unlike *Cblb*$^{-/-}$ mice, it is interesting to note that no overt autoimmunity developed in any of the *Cbl*$^{-/-}$ mice. However, mice deficient in both CBL and CBL-B show increased development of autoimmunity and mortality compared with the single-knockout mice[77]. Peripheral T cells from the *Cbl*$^{-/-}$*Cblb*$^{-/-}$ mice are hyperproliferative and have increased cytokine production, similar to a T-cell memory phenotype. Although TCR signalling pathways were not enhanced, *Cbl*$^{-/-}$*Cblb*$^{-/-}$ T cells have impaired ligand-induced TCR downregulation, which leads to sustained TCR signalling and the development of autoreactive T cells. The authors speculate that CBL and CBL-B could promote the destabilization of the T-cell synapse soon after T-cell activation. This hypothesis is supported by the observation that ionomycin-induced anergic T cells, with sustained expression of E3 ubiquitin ligases such as CBL-B, also have difficulties in forming competent T-cell synapses[54]. Surprisingly, as *Cbl*$^{-/-}$*Cblb*$^{-/-}$ mice efficiently developed both CD4^{+} and CD8^{+} T cells in the absence of MHC expression, CBL and CBL-B could also help to confer MHC dependence on developing thymocytes[78]. This function ensures not only that peripheral T cells will be able to clear pathogens in an MHC-dependent manner, but also that the mechanisms of peripheral tolerance will be able to regulate peripheral T cells.

GRAIL. GRAIL is a 428-amino-acid type 1 transmembrane protein that contains an extracellular protease associated (PA) domain and cytosolic coiled-coil and RING-finger domains. mRNA encoding GRAIL was first isolated from T-cell clones 4 hours after stimulation with peptide–MHC complexes without CD28 co-stimulation, and upregulation of GRAIL expression has also been observed in anergic T cells generated by other systems, including treatment of T cells with ionomycin *in vitro* and administration of SEB to TCR-transgenic mice for the induction of adaptive tolerance[55,79]. GRAIL is mainly found associated with the recycling endosomal compartment, and there are other family members identified in the human genome that contain the same domain organization, suggesting that there may be some functional redundancy[80] (FIG. 3).

The RING-finger domain of GRAIL is of the C3H2C3 type, and it has been shown to act as an E3 ubiquitin ligase *in vitro* with several E2 ubiquitin transferases[55]. The coiled-coil region was found to interact with otubain-1, which is a ubiquitin isopeptidase of the ovarian tumour (OTU) superfamily. Expression of otubain-1 enhanced GRAIL autoubiquitylation and proteasomal degradation, whereas a splice variant of otubain-1 that lacks the core of the OTU domain stabilizes GRAIL protein[81]. Interestingly, because otubain-1 itself contains catalytic activity only towards isolated, branched polyubiquitin chains, its binding to GRAIL promotes GRAIL autoubiquitylation and degradation presumably by inhibiting the activity of a separate canonical deubiquitylating enzyme. Subsequent analysis found that the alternative splice form of otubain-1 promoted the recruitment of USP8 (ubiquitin-specific peptidase 8) for the deubiquitylation of GRAIL[81].

Bone-marrow chimeric mice with T cells that express GRAIL or the splice variant of otubain-1 have markedly reduced proliferation and IL-2 production. By contrast, bone-marrow chimeric mice expressing either a dominant-negative mutant of GRAIL that lacks a functional RING-finger domain or wild-type otubain-1 have impaired T-cell anergy induction, suggesting that GRAIL acts at a key step to determine T-cell activation versus anergy induction[79,81]. Therefore, among the E3 ubiquitin ligases associated with T-cell anergy, GRAIL is unique in its ability to recapitulate anergy owing to its expression alone. Recent evidence also indicates that GRAIL, like CBL-B, can regulate T-cell activation by modulating the dynamics of the actin cytoskeleton[82]. Instead of controlling the GTP loading of RHO-family members, GRAIL polyubiquitylates and stabilizes the RHO-family GDP-dissociation inhibitor (RHOGDI)[82]. Higher levels of cellular RHOGDI in turn sequester more RHO-family small GTPases away from the membrane regardless of their GTP-loading state, thereby preventing reorganization of actin into stress fibres or focal adhesions[83]. These data provide evidence for a model of incomplete immunological synapse formation in GRAIL-expressing T cells, resulting in impaired T-cell activation.

OTU domain
(Ovarian tumour domain). A domain that is found in a large family of proteins characterized by the presence of a putative catalytic triad of cysteine proteases. Several of these proteins are known to function as deubiquitylating or ubiquitin-modifying enzymes.

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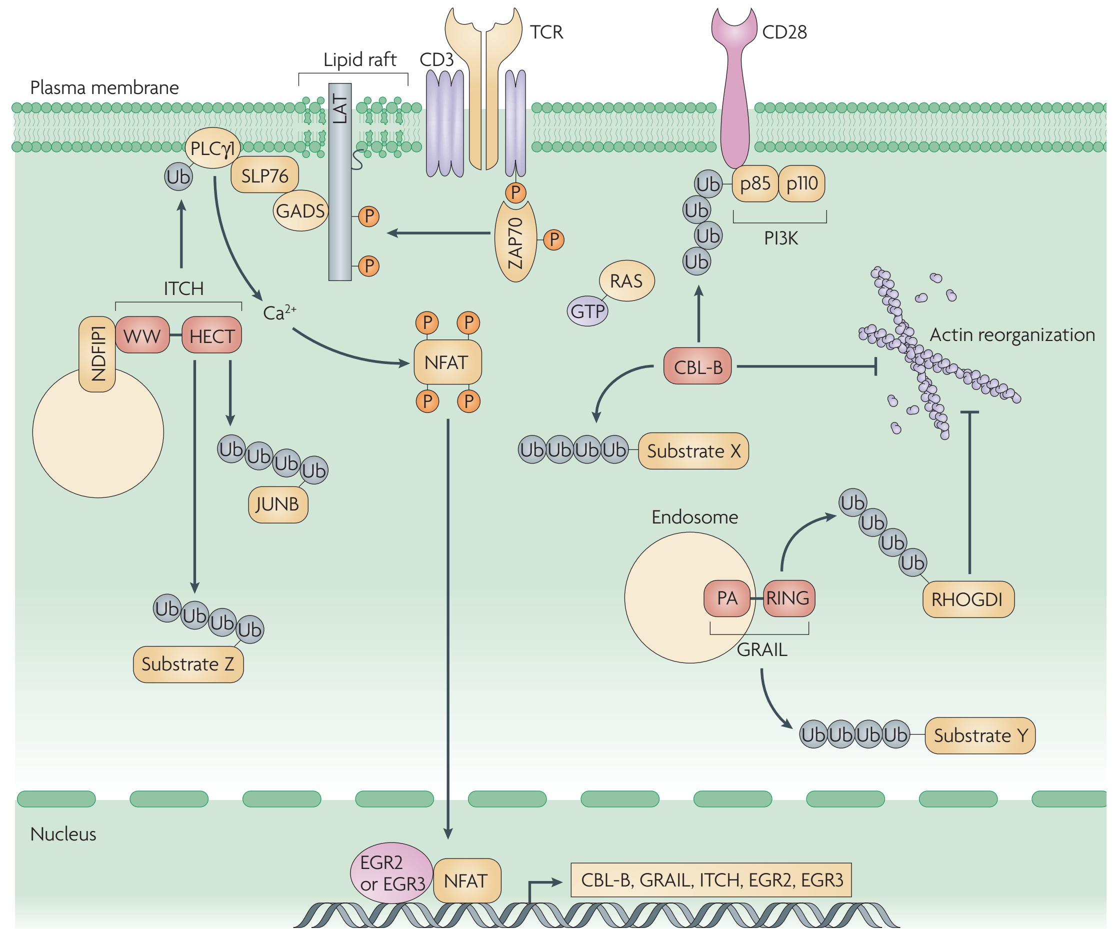

Figure 4 | **A model for regulation of T-cell activation by three E3 ubiquitin ligases following anergy induction.** In response to the presence of NFAT (nuclear factor of activated T cells) in the nucleus without contribution from other transcription factors activated by the MAPK (mitogen-activated protein kinase) signalling pathway, a genetic anergic signal is imparted on the T cell. Specifically, NFAT causes the upregulation of expression of three E3 ubiquitin ligases, CBL-B (Casitas B-lineage lymphoma B), GRAIL (gene related to anergy in lymphocytes) and ITCH (itchy homologue E3 ubiquitin protein ligase), as well as of the transcription factors early growth response 2 (EGR2) and EGR3. These E3 ubiquitin ligases, unrelated at the polypeptide level, target different signalling nodes that are essential for T-cell activation. ITCH (along with its related E3 ubiquitin ligase NEDD4 (neural precursor cell expressed, developmentally downregulated 4)) has been shown to monoubiquitylate both phospholipase Cγ1 (PLCγ1) and protein kinase Cθ (PKCθ) for lysosomal degradation and termination of signalling downstream of linker for activation of T cells (LAT). CBL-B polyubiquitylates the p85 subunit of PI3K (phosphoinositide 3-kinase) and prevents PI3K from associating with CD28, thus attenuating this co-stimulatory signalling cascade. CBL-B expression also leads to deficient T-cell synapse formation by inhibiting actin reorganization downstream of the RHO-family small GTPase VAV1. GRAIL has also been shown to modulate the activation of RHO-family members by polyubiquitylating and stabilizing the RHO inhibitor RHOGDI (RHO guanine-dissociation inhibitor). Thus, the combined effects of ITCH, CBL-B and GRAIL serve as a potent inhibitory signal to prevent T-cell activation after stimulation through the T-cell receptor (TCR), as could occur during strong binding to self-protein–MHC complexes in the periphery. GADS, GRB2-related adaptor protein; HECT, homologous to E6-AP carboxy terminus; NDFIP1, NEDD4-family interacting protein 1; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; PA, protease-associated domain; RING, really interesting new gene; ZAP70, ζ-chain-associated protein kinase of 70 kDa.

Agouti locus
The agouti locus on mouse chromosome 2 determines the coat colour of a mouse by regulating the synthesis of yellow pigment by hair melanocytes. Mutations in this locus are also linked to immunological defects and the development of obesity and neoplasms.

C2 domain
A versatile interaction domain that binds a wide variety of substrates including various lipids, calcium ions and phosphotyrosine residues.

WW domain
A protein–protein interaction module that contains two conserved tryptophan (W) residues that are 20–22 amino acids apart and that interacts with proline-rich motifs.

ITCH. ITCH is an 864-amino-acid cytosolic E3 ubiquitin ligase that is encoded by the agouti locus. When mutated in a^{18H} mice, *Itch* is responsible for the development of a systemic lymphoproliferative disease that is characterized by enlarged secondary lymphoid organs and constant itching of the skin[84]. Unlike the other E3 ubiquitin ligases CBL-B and GRAIL, ITCH uses a HECT (homologous to E6-AP carboxy terminus) domain for the recruitment of E2 ubiquitin transferases. It also has an amino-terminal protein-kinase-C-related C2 domain and four centrally located WW domains for low-affinity binding to proline-rich regions. The C2 domain directs ITCH to endosomes, thereby leading to the co-localization of ITCH with most GRAIL molecules[85].

Following TCR stimulation, the recruitment of PLCγ1 to LAT generates both inositol-1,4,5-trisphosphate ($InsP_3$) and DAG by catalysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) (FIG. 4). Formation of these two intermediates leads to rapid calcium flux (through $InsP_3$) and the recruitment

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of C1-domain-containing proteins, such as PKCθ and RASGRP1, to internal membranes (through DAG). The upregulation of expression of ITCH, and its closely related E3 ubiquitin ligase NEDD4 (neural precursor cell expressed, developmentally downregulated 4), after ionomycin-induced clonal T-cell anergy was found to promote lysosomal degradation of PLCγ1 and PKCθ by monoubiquitylation[54]. This action effectively abrogates both the calcium flux and signalling that are required for the organization of proteins at the immunological synapse. The essential nature of the LAT–PLCγ1 interaction for rapid dissemination of the T-cell activation signal is highlighted by the convergence of three processes in anergic cells — LAT hypopalmitoylation, PLCγ1 and PKCθ degradation, and DAG signal attenuation. Therefore, it seems probable that this signalling node downstream of the TCR, illuminated by multiple model systems, is regulated by the anergy-associated E3 ubiquitin ligases and serves as a crucial fulcrum that determines the balance between anergy induction and T-cell activation (FIG. 4).

In addition to ITCH-mediated regulation of TCR-proximal events, studies have shown that ITCH might be involved in the reduced IL-2 production of anergic T cells by acting on JUNB[86]. Clues for how this regulation occurs came from studies of *Itch*$^{-/-}$ T cells, which revealed that members of the JUN family of transcription factors are among the key substrates of ITCH, marking them for degradation. *Itch*$^{-/-}$ T cells showed an increase in IL-4 production and a general skewing towards a T_H2-cell phenotype, due to prolonged JUNB stability and DNA-binding activity[87]. ITCH itself was found have increased ubiquitylation activity towards both JUNB and JUN following phosphorylation of ITCH by the MEKK1–JNK1 (MAPK/ERK kinase kinase 1–JUN N-terminal kinase 1) pathway downstream of TCR stimulation[88]. The phosphorylation of three serine/threonine residues of ITCH by JNK1 induces a conformational change that relieves autoinhibitory binding between the WW domains and the HECT domain, whereas tyrosine phosphorylation by FYN reduces its ability to bind JUNB[89,90]. Although ITCH mainly resides outside the nucleus and the JUN family molecules are located inside in the nucleus, ubiquitin-mediated fluorescence complementation studies showed ubiquitylated JUN, mediated by ITCH, present in cytosolic lysosomal compartments[91]. However, how and where ITCH-mediated ubiquitylation of JUN transcription factors occurs has yet to be clearly established. Also, due to the redundancy of four FOS-family members and three JUN-family members, it cannot be certain that ITCH-mediated regulation of JUNB or JUN could completely abrogate AP1 formation. The finding that ITCH was a factor involved in determining T_H1-cell lineage commitment is intriguing because T-cell anergy was initially characterized using T_H1-cell clones. Interestingly, a recent study reported that ITCH activation induced by the MEKK1–JNK1 pathway was required for the induction of tolerance in T_H2 cells both *in vitro* and in a mouse model of allergy[92].

In addition to phosphorylation-induced activation, ITCH activity also depends on accessory binding proteins. NDFIP1 (NEDD4-family interacting protein 1; also known as N4WBP5) is a transmembrane protein that is localized to the Golgi apparatus and that binds to the WW domains of the NEDD4-family members (including NEDD4 and ITCH) and is proposed to function as an adaptor for ubiquitylated targets of the NEDD4 family[93]. Interestingly, *Ndfip1*$^{-/-}$ mice recapitulate the phenotype of *Itch*$^{-/-}$ mice, being characterized by increased T-cell proliferation and T_H2-type cytokine production owing to the prolonged presence of JUNB in the nucleus[94]. The expression of NDFIP1 was found to be upregulated following T-cell activation and to associate with ITCH, which then promoted the degradation of JUNB. Further refinement of the NDFIP1–ITCH interaction may help to clarify the mechanisms of JUNB ubiquitylation. NDFIP1 may also have a pivotal role in clonal anergy as its related family member and NEDD4 binding partner NDFIP2 (also known as N4WBP5A) is upregulated following T-cell activation in a calcineurin-dependent manner[95].

Ubiquitin modification and binding

The main questions that invariably arise following the cloning of an E3 ubiquitin ligase include: what are its substrates? What context allows the E3 ubiquitin ligase to bind its substrates? And how does ubiquitylation regulate substrate function? In addition, the immense flexibility and reversibility of the ubiquitin–proteasome system has recently begun to be appreciated as a measure of self-containment. One example is the ability of E3 ubiquitin ligases to be regulated in the same manner as their substrates. E3 ubiquitin ligases can serve as substrates for other E3 ubiquitin ligases, as shown by ITCH- and NEDD4-mediated ubiquitylation of CBL and CBL-B[96]. Conversely, E3 ubiquitin ligases are subject to some level of autoubiquitylation, in which ubiquitin is transferred to the lysine residues of the E3 ubiquitin ligase itself. This process can occur at a low rate due to the absence of a substrate, or it can be a directed event used to control the protein levels of an E3 ubiquitin ligase. This scenario is the case for the regulation of both GRAIL, in which otubain-1 recruits USP8 to control the levels of autoubiquitylated GRAIL, and ITCH, which recruits USP9X (USP9, X-linked; also known as FAM) to control its autoubiquitylation[81,97]. It is notable that both GRAIL and ITCH recruit proteins that simply remove added ubiquitin molecules rather than modifying their own intrinsic autoubiquitylation reaction, suggesting that E3 ubiquitin ligases could have a steady-state level of binding of E2 ubiquitin transferases and transferring ubiquitin without regard for the presence of a substrate.

Besides deubiquitylating enzymes that remove ubiquitin, there are many classes of ubiquitin-binding proteins that are responsible for interpreting the attached ubiquitin signal and handling the substrate accordingly[98]. Suppressor TCR signalling 1 (STS1) and STS2 (also known as TULA and UBASH3A) are cytosolic proteins that contain ubiquitin associated (UBA) domains, which are capable of binding ubiquitin. Peripheral T cells from

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mice that lack STS1 and STS2 are hyper-responsive to TCR stimulation, have increased T-cell signalling beginning at ZAP70 (ζ-chain-associated protein kinase of 70 kDa) and are more susceptible to the induction of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis[99]. STS1 and STS2 antagonize CBL-mediated downregulation of cell-surface growth factor receptors[100], but there is uncertainty about whether in haematopoietic cells STS1 and STS2 act as activators or inhibitors of T-cell activation, possibly through regulation CBL[99,101].

Concluding remarks

The past year has yielded several important contributions that help to refine our understanding of the mechanisms involved in the induction of T-cell clonal anergy. Based on the current evidence regarding ubiquitylation and anergy that is presented here, we propose that these seemingly disparate E3 ubiquitin ligases act at multiple nodes of T-cell activation, from proximal TCR signalling at the cell surface to endocytic membranes trafficking to transcription factors, thereby regulating signal processing and dissemination (FIG. 4). Although the penetrance and redundancy of the individual E3 ubiquitin ligases is currently unclear, each enzyme could function at a different level of the signal network to exert local control of T-cell activation. One level may be sufficiently significant to diminish an aspect of T-cell activation, such as proliferation, but numerous layers of regulation by a myriad of E3 ubiquitin ligases could be the preferred mechanism for ensuring suitable control over T-cell activation and tolerance.

However, several important questions remain to be resolved. First, how do the alterations in T-cell signalling pathways that emanate from the TCR fit together with the identified E3 ubiquitin ligases during the induction and maintenance of T-cell anergy? Is there a precise kinetic order of events that must be achieved or are they completely separate events? As there are hundreds of E3 ubiquitin ligases responsible for ubiquitin-dependent regulation of a vast majority of the human proteome, can CBL-B, GRAIL and ITCH regulate more substrates than are currently known for them? In addition, are other members of the ubiquitin–proteasome system, such as deubiquitylating enzymes or ubiquitin-binding proteins, differentially upregulated in T-cell anergy and function in conjunction with CBL-B, GRAIL and ITCH? Finally, a priority in the field must be to translate the many descriptive events documented so far into treatments that can turn off unwanted antigen-specific T cells *in vivo* and thus mitigate the effects of debilitating immunological quandaries such as autoimmune disease.

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Acknowledgements
We apologize to all those researchers who have made contributions to the field of T-cell tolerance and anergy but were not discussed due to space restrictions. We thank L. Su and the editors for discussions and critical reading of the manuscript. Supported by the National Institutes of Health, USA, grants CA65237 and DK078123.

Competing interests statement
The authors declare no competing financial interests.

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