Cell Death and Differentiation (2008) 15, 1103–1112
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Review

Itch: a HECT-type E3 ligase regulating immunity, skin and cancer

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The HECT-type E3 ubiquitin ligase (E3) Itch is absent in the non-agouti-lethal 18H or *Itchy* mice, which develop a severe immunological disease, including lung and stomach inflammation and hyperplasia of lymphoid and hematopoietic cells. The involvement of Itch in multiple signaling pathways and pathological conditions is presently an area of extensive scientific interest. This review aims to bring together a growing body of work exploring Itch-regulated biological processes, and to highlight recent discoveries on the regulatory mechanisms modulating its catalytic activity and substrate recognition capability. Our contribution is also an endeavor to correlate Itch substrate specificity with the pathological defects manifested by the mutant *Itchy* mice.
Cell Death and Differentiation (2008) **15,** 1103–1112; doi:10.1038/cdd.2008.60

The E3 protein ubiquitin ligase (E3) Itch, or atrophin-1 interacting protein 4 (AIP4, hereafter referred to as Itch), was originally identified through genetic studies aimed to examine the *agouti* locus, whose mutation results in coat color alterations in mice.[1] The 18H mutation, which is associated with a darker coat, arises from a radiation-induced chromosomal inversion that deletes 18 and 20 base pairs from the proximal and distal inversion breaks, respectively. The inversion disrupts the expression of *agouti* and *Itch* genes.[1]

The *Itch* gene encodes 854 amino acids with a relative molecular weight of 113 kDa. Itch is a monomeric protein, which belongs to the homologous to E6-AP carboxy terminus (HECT)-type family of E3s, whose modular structural organization consists of an N-terminal Ca^{2+}-dependent phospholipid-binding C2 domain, multiple protein–protein interaction WW domains, and a C-terminal HECT domain.[2] The latter coordinates with cognate E2 ubiquitin-conjugating enzyme (E2), and contains an evolutionary conserved cysteine, which forms thioester complexes with ubiquitin, before final attachment of ubiquitin to the target proteins.[3] Itch possesses four WW domains and a unique proline-rich motif (PRR) located between residues 195 and 246, displaying important regulatory functions. Similarly to other HECT-type E3s, Itch WW domains most commonly recognize the Pro-rich PPXY (PY) consensus sequence, though they also interact with phospho-Ser/phospho-Thr followed by a Pro residue. In addition, atypical interactions with unrelated modular domains in the substrate[4–6] or adaptor proteins[7,8] have been described.

Although a small fraction of Itch displays a perinuclear distribution overlapping with the trans-Golgi network, the protein is predominantly associated with early and late endosomal compartments and lysosomes.[5,9] Localization of Itch to the endocytic vesicles is mediated by the C2 domain.

The non-agouti-lethal 18H or *Itchy* mice display severe immune and inflammatory defects and manifest a persistent scratching of the skin.[1] On the C57BL/6J background, Itch deficiency results in spontaneous development of a late onset and progressively lethal systemic autoimmune-like disease, characterized by lymphoproliferation in the spleen, lymph nodes and medulla of the thymus, and by chronic pulmonary interstitial inflammation. Death, occurring at 6–8 months of age, is likely caused by hypoxia, associated with pulmonary inflammation and alveolar proteinosis.

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Keywords: ubiquitin E3 ligase; apoptosis; JunB; p53 family members; Notch
Abbreviations: AICD, activation-induced cell death; c-FLIP$_L$, cellular FLICE-inhibitory protein; EGFR, epidermal growth factor receptor; HECT, homologous to E6AP COOH-terminus; JNK, jun N-terminal kinase; ICD, intracellular domain; IL, interleukin; Mekk1, mitogen and extracellular kinase kinase 1; PLC-γ1, phospholipase C-γ1; PKC-θ, protein kinase θ; PY motif, proline-rich sequence; RING, really interesting new gene; Smurf, SMAD ubiquitylation regulatory factor; *Su(dx), suppressor of deltex*; TCR, T-cell receptor; TGFβR, transforming growth factor-β receptor; TNFα, tumor necrosis factor-α; TH, T-helper lymphocyte
Received 25.2.08; revised 26.3.08; accepted 26.3.08; Edited by P Nicotera

Antigen-stimulated naïve CD4^{+} T-helper (T_H) cells can differentiate into two distinct subsets of effector cells, T_H1 and T_H2, defined by specific cytokine profiles and distinct immune regulatory functions. T_H2 cells typically produce interleukin-4 (IL-4) and counteract extracellular pathogens. Dysregulation of the T_H2-type response is typically responsible for development and maintenance of asthmatic and allergic diseases such as allergic airway inflammation and atopic dermatitis. In response to anti-CD3 and anti-CD28 costimulation, the *Itch* null T lymphocytes display increased production of T_H2 cytokines (e.g. IL-4 and IL-5), causing biased differentiation of CD4^{+} cells into T_H2 cells and chronic activation.[10] As a consequence, the *Itchy* null mice exhibit a higher level of T_H2-dependent immunoglobulin (Ig) G1 and E subtypes than their normal counterpart.[10]

It is relevant to point out that the phenotypic characterization of the *Itchy* mice on the JU/Ct or C57BL/10 background has revealed that the bias toward production of T_H2-derived cytokines is not the only determining factor in the development of the autoimmune disease.[11] As recently reported by Parravicini *et al.*,[11] Itch disruption in $\alpha\beta$ and $\gamma\delta$ T cells causes expansion of B1b lymphocytes leading to IgM elevation, and initiates IgE production, respectively. The expansion of B1b cells and elevated Ig levels correlated with itching and other inflammatory symptoms. The availability of different animal models of Itch deficiency will certainly contribute to unveil the complex molecular defects underlying the autoimmune pathology.

With few exceptions, Itch typically regulates the stability of both transmembrane receptors through canonical monoubiquitylation or multiubiquitylation, and intracellular substrates through polyubiquitylation, driving them to lysosomal and proteasomal degradation, respectively. Proteolysis-independent ubiquitylation events have been also ascribed to the E3 activity of Itch.[6,12] A growing number of new substrates and regulatory pathways along with the functional versatility of Itch have been recently brought to light. By illustrating Itch substrate specificities, here, we review the different biological processes involving its E3 activity. The main features of Itch substrates are summarized in Table 1.

Itch Substrates in Relation to Their Biological Functions

Regulation of the immune response. Notably, several Itch substrates are central players or modulators of the immune response. By providing a portrait of Itch protein targets, we intend to bring together Itch substrate specificity with its ability to regulate the immune system, and ultimately, with the pathological defects developed by the mutant *Itchy* mice.

Jun family members: the molecular basis underlying Itch-regulated differentiation of T lymphocytes. The identification of c-Jun and JunB as two Itch protein substrates[8,10,13] has shed light on the molecular basis underlying the immunological phenotype of the *Itchy* mice. JunB and c-Jun contain PY or PXY motifs that serve as binding site for Itch WW domains. Ubiquitin-conjugated c-Jun is mainly localized to lysosomal vesicles and its degradation appears to involve both the lysosomal and the proteasomal pathways.[13] As a result of Itch-mediated canonical ubiquitylation of its substrate JunB, IL-4 promoter occupancy by this transcription factor is greatly reduced upon T-cell receptor (TCR) stimulation.[10] As JunB protein selectively accumulates in T_H2 cells and is involved in T_H2 cell differentiation through the transcriptional regulation of IL-4 and IL-5 promoters,[14,15] it represents a good candidate mediating the dysregulation of CD4^{+} T-lymphocyte function observed in the *Itchy* mice. Under physiological conditions, Itch-induced degradation of JunB maintains the levels of IL-4 at low concentrations, thus attenuating the T_H2 differentiation response (Figure 1). On the other hand, aberrant expression of JunB, such as a result

Table 1 Main features of Itch substrates

Substrate	Function	Biological outcome	Regulators/ adaptors	References
c-Jun	Transcription factor	Regulation of T_H2 cell differentiation/anergy	JNK, N4BP1	8,10,12,49,50
Jun-B	Transcription factor	Regulation of T_H2 cell differentiation/anergy	JNK, Fyn, Ndfip1	10,12,49,50,54,60
PLC-γ1	Phospholipase	Regulation of T-cell anergy	Unknown	16
PKC-θ	Kinase	Regulation of T-cell anergy	Unknown	16
Notch	Transcription factor	Regulation of autoimmunity	Numb	4,20
Gli	Transcription factor	Repression of Hedgehog signaling	Numb	61
Deltex	Regulator of Notch signals	Regulation of autoimmunity	Unknown	22
Smad2	Receptor-activated Smad	Activation of TGFβR signaling	Unknown	11
TIEG1	Transcription factor	Activation of TGFβR signaling	Unknown	27
P73	Transcription factor	Regulation of apoptosis, neural development, cancer	N4BP1	34,8
P63	Transcription factor	Regulation of apoptosis, epithelial development, cancer	N4BP1	35,8
c-Flip	Apoptosis inhibitory protein	Regulation of apoptosis	JNK	43
ErbB4	Growth factor receptor	Epithelial kinase receptor, cancer	Unknown	48
Endophilin A1	Protein of the endocytic machinery	Regulation of EGFR endocytosis	Unknown	9
CXCR4	Chemokine receptor	Agonist-dependent sorting of G protein-coupled receptors	Unknown	5
Hsr	Protein of the endocytic machinery	Regulation of cargo sorting	Unknown	5
TRPV4, TRPC4	Cation channels	Regulation of channel recycling and abundance at the cell surface	Unknown	6
Itch	E3 ubiquitin ligase	Control of protein stability, potential regulation of E3 catalytic activity	USP9X/FAM	8,49,50,55

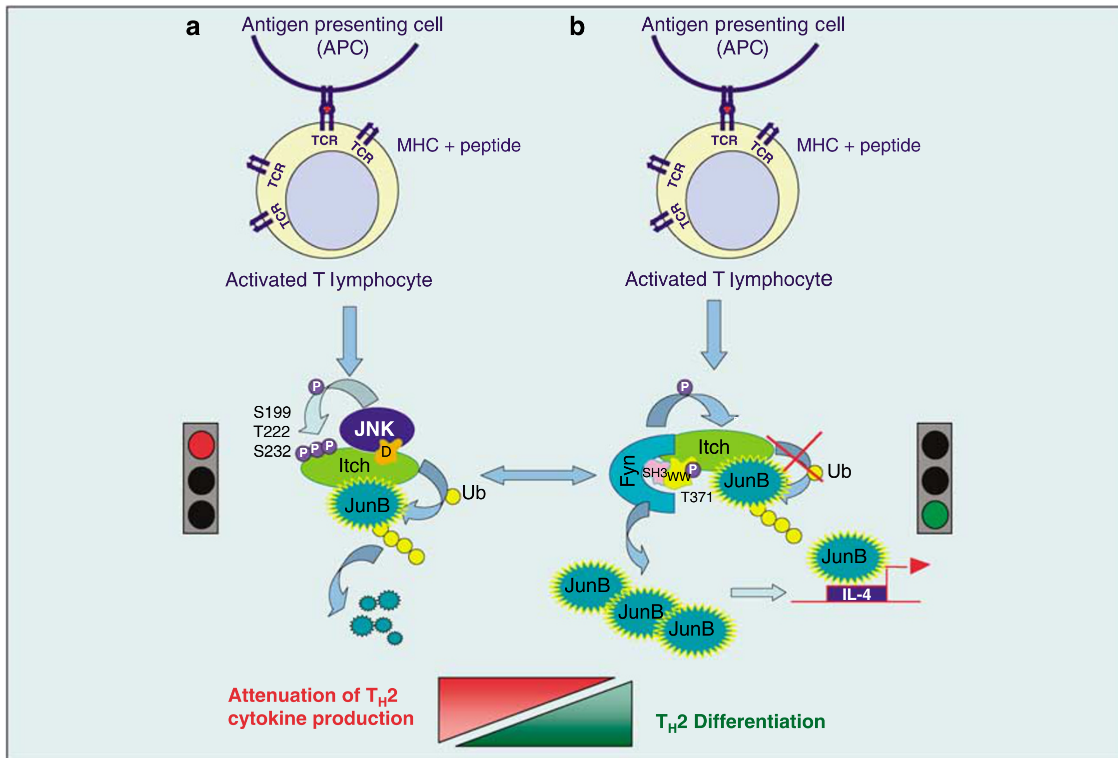

Figure 1 Itch regulation upon TCR engagement. Interaction of a TCR with its cognate antigenic peptide bound to an MHC class II molecule activates a $CD4^+$ T-cell response. (**a**) Attenuation of a T_H2 response is mediated by the JNK/MAPK signaling. T-lymphocyte stimulation initiates the JNK/MAPK signaling cascade culminating in JunB degradation through the phosphorylation-dependent activation of Itch. Serine/threonine phosphorylation of Itch attenuates the polarization of $CD4^+$ T-lymphocytes into T_H2 effector cells and could prevent JunB from driving IL-4 production in response to strong T-cell activating signals. (**b**) Potentiation of a T_H2 response is promoted by the Scr kinase Fyn. An additional level of regulation of Itch activation in response to TCR stimulation is achieved through the Fyn tyrosine kinase signaling pathway. Fyn-catalyzed Tyr371 phosphorylation of Itch prevents its association with JunB. As a consequence of reduced substrate recognition, ubiquitin conjugation and proteasomal proteolysis of JunB are diminished, and augmented IL-4 production accelerates T_H2 differentiation. Thus, JunB turnover is finely controlled by upstream kinases through counterbalancing serine/threonine *versus* tyrosine phosphorylation of Itch

of *Itch* mutation or downregulation, polarizes T cells toward the T_H2-differentiated features, ultimately causing T-cell hyperproliferation and abnormal allergic responses, including elevated serum IgG1 and IgE.[10] The loss-of-function approach, in which JunB is selectively deleted in T-lymphocytes complements and functionally supports the observation that, enhanced JunB activity due to either *Itch* loss[10] or JunB overexpression in mice,[14] leads to a shift of $CD4^+$ T cells toward the T_H2 phenotype. JunB-deficient mice indeed exhibit an impaired allergen-induced airway inflammation.[15]

PLC-γ and PKC-θ: Itch as a T-cell anergy determinant. Induction and maintenance of peripheral immune tolerance is accomplished through the activation of clonal T-cell anergy, a process that prevents the ability of autoreactive lymphocytes to differentiate and proliferate in response to a self-antigen peptide. Anergy induction is triggered by stimulation of the TCR in the absence of the CD28 costimulatory signal on the surface of the antigen-presenting cell (APC), and is typically characterized by the inability of T cells to produce IL-2, even following the re-stimulation of the TCR and the costimulatory receptor. The establishment of the anergy state requires the Ca^{+2}/calcineurin-mediated sustained activation of the NFAT transcription factor, in the absence of AP-1 cooperation. Maintenance of T-cell anergy is achieved through the ubiquitin-mediated degradation of key signaling molecules, which ultimately disrupt the integrity of the immunological synapse after restimulation of anergic cells. This aspect has been reviewed at length elsewhere.[16] The immunological synapse, a supramolecular complex at the T cell/APC interface, mainly composed of adhesion molecules and TCR signal transduction machinery, is crucial for productive T-cell signaling and proliferation.

Itch regulates T-cell anergy maintenance by targeting the phospholipase C-γ1 (PLC-γ1) and the protein kinase θ (PKC-θ), two key signaling molecules induced by calcium/calcineurin signaling, for monoubiquitylation. According to the model proposed by Heissmeyer *et al.*,[17] Itch mRNA levels are induced during T-cell anergy in an NFAT-dependent manner. After restimulation of anergic cells, newly expressed Itch redistributes from the cytosol to the endosomal compartment, where it can associate with PLC-γ1 and PKC-θ, which are adjacent to the immunological synapse. Following Itch-mediated monoubiquitylation, PLC-γ1 and PKC-θ undergo endosomal sorting and trafficking into the lysosome for protein degradation. Decrease of PLC-γ1 and PKC-θ abundance is thought to shorten the lifespan of the immunological synapse.

The inability to sustain stable APC contacts reduces the antigen response of anergic cells and induces T-cell unresponsiveness after TCR engagement. In addition, Itch may regulate anergy and autoimmunity by catalyzing the ubiquitin-dependent degradation of the Jun family members, thus contributing to AP-1 inactivation, and indeed the kinetics of CD3 *versus* CD3, and CD28 JunB expression in $CD4^+$ T cells are different.

The critical role played by Itch in peripheral immune tolerance *in vivo* became evident from the observation that T cells from Itch deficient mice are resistant to anergy induction and do not show downregulation of PLC-γ1 and PKC-θ following anergy-stimulating conditions.[17] Besides the hampering effect exerted on signal transduction at the synapse, Itch may regulate anergy and autoimmunity by catalyzing the ubiquitin-dependent degradation of the Jun family members, thus contributing to AP-1 inactivation. JunB is indeed responsible for AP-1-dependent transactivation of the IL-2 promoter in T_H1 cells.

Therefore, the inability to induce anergy may be one of the molecular mechanisms underlying the autoimmune symptoms, such as splenomegaly and lymphocyte infiltration, manifested by the *Itch* deficient mice.

By using an *in vivo* mouse model of antigen-induced tolerance, Venuprasad *et al.*[18] have recently proposed that Itch can also function as a crucial tolerogenic modulator of T_H2 cells. Under T_H2 conditions, tolerized *Itch* null T cells are more resistant to anergy induction. They indeed do not downregulate the Jun family members, and thereby undergo sustained transcriptional activation of the IL-4 promoter.[18] As a result, the *Itch* null mice are thought to develop the autoimmune symptoms, such as the allergic inflammatory lesions affecting the lung.

Notch receptors: highlighting the molecular basis of the Itchy autoimmune disease. The failure to establish peripheral tolerance due to enhanced levels of Jun family members, PLC-γ1 and PKC-θ, only in part, accounts for the autoimmune disease observed in the *Itch* mutant mice. The discovery of Notch as a target of Itch-catalyzed ubiquitylation has provided an additional level of complexity to the regulation of the immune response by Itch[4]. Notch proteins (four in mammals) are evolutionarily conserved type I transmembrane receptors, which mainly function in specifying cell fate decision during differentiation and morphogenesis. Depending on tissue and context, Notch activation can either promote or restrict cell fate determination. During lymphoid development, Notch proteins play an essential role in the induction of T cell and B cell lineages and specification of T-cell effector fates, including T_H2 differentiation (reviewed in Osborne and Minter[19] and references therein; Figure 2). In addition, Notch promotes activation and cell survival of mature peripheral T lymphocytes by upregulating the expression of antiapoptotic proteins such as Bcl-2 and inhibitor of apoptosis proteins (IAPs), and through the activation of AKT (Figure 2).[20]

The ubiquitin/proteasome degradation pathway plays a critical role in regulating Notch signal transduction. Upon ligand binding, Notch proteins undergo a complex proteolytic maturation process, which culminates in the release of the intracellular domain (ICD). Cleavage of the receptor is triggered when the extracellular domain binds to a canonical ligand on a neighboring cell, or upon activation of $CD4^+$ T lymphocytes. The transcriptionally active ICD translocates to the nucleus, where it functions as coactivator to modulate transcription of Notch target genes. Itch-mediated polyubiquitylation of the intracellular portion of membrane-tethered Notch1 results in the degradation of the ICD following

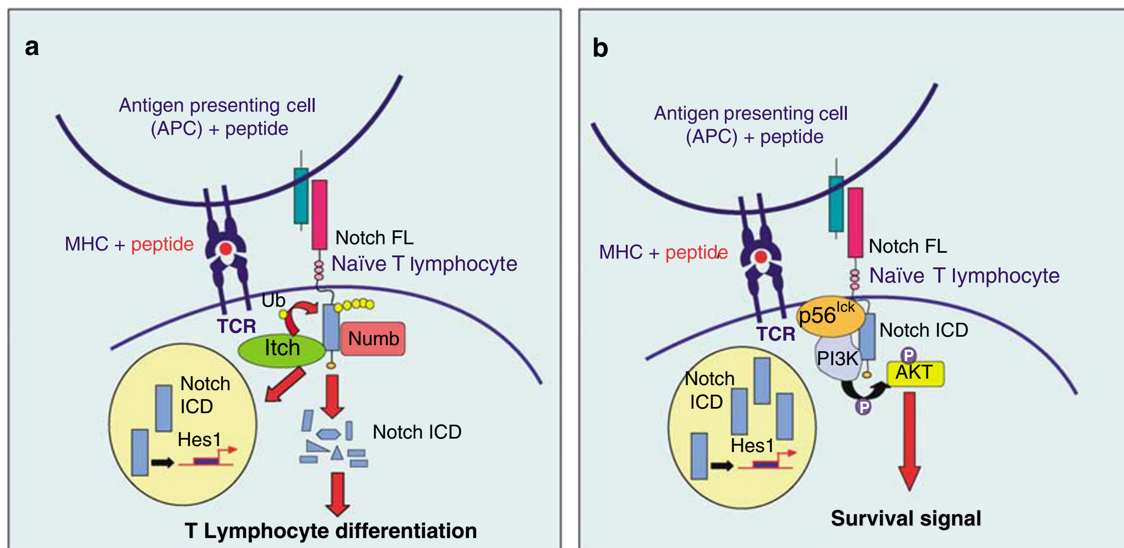

Figure 2 A model for Itch contribution to Notch-regulated lymphoid development. The Notch receptors regulate cell fate decisions during T-cell differentiation and activation. As a result of ligand binding to the extracellular domain, Notch proteins undergo a complex processing mechanism, culminating with the release of the transcriptionally competent ICD from the membrane. (**a**) Following receptor activation, Itch-mediated ubiquitylation of Notch results in the degradation of the ICD, thus, in turn, antagonizing Notch nuclear activity. The protein adaptor Numb contributes to modulate the endocytic ubiquitin-dependent proteolysis of Notch1 and, thus, to finely tune the specification of T-cell fates determined by Notch signaling. (**b**) Under physiological conditions, Notch promotes the activation and cell survival of mature peripheral T lymphocytes, by upregulating the expression of a number of antiapoptotic proteins, including AKT. Unbalanced Notch turnover, due to Itch mutation or downregulation, would increase Notch signaling and thereby amplify survival signals leading to abnormal immune responses

receptor activation (Figure 2).[4,21] Due to the lack of PY motifs, Notch1 is atypically recruited by Itch through the N-terminal ankyrin repeat-containing region of the ICD. As discussed later in this review, the binding affinity of Itch for Notch and the resulting receptor degradation are finely tuned by the action of the vertebrate homolog of *Drosophila* Numb.[21] An additional level of regulation of Notch activity is accomplished through Itch-mediated degradation of Deltex, which positively controls Notch signaling pathway. Modification of Deltex with lysine29-linked polyubiquitin chains labels it for the endocytic pathway, and ultimately, for lysosomal proteolysis.[22]

Notch has been proposed as one of the most relevant Itch target, responsible for the autoimmune phenotype manifested by the *Itchy* mice. *In vivo* studies using an activated *Notch1* transgene specifically expressed in developing thymocytes showed that increased Notch1 signaling results in a chronic autoimmune-like disease, resembling the *Itchy* mouse symptoms.[23] Even more remarkably, the *Itchy* mutant mice expressing the activated *Notch1* transgene, display even more severe pathological lesions including splenomegaly, hepatomegaly and lymphadenopathy, chronic inflammation as well as a significant earlier disease onset. Notch signaling positively regulates the expression of IL-4 by $CD4^+$ T cells. It is, therefore, likely that the hyperproliferative and inflammatory phenotype manifested by the *Itchy* mice is worsened by further production of IL-4 due to increased expression of Notch in the double-mutant mice. In addition, the mutations in concert yielded a novel phenotype including a perturbation of T-cell development (e.g. negative or positive selection). The severity of the disease correlated with increased Notch1 protein levels, leading to augmented AKT signaling pathway. The survival signal generated through phosphorylated AKT would then allow the persistence of pathological cells that can initiate the autoimmune disease.[24]

Notch signaling and its regulation pathways are evolutionarily conserved mechanisms. The *Drosophila* mutant *Suppressor of deltex* (*Su*(*dx*)) was originally identified as a suppressor of the wing vein thickening caused by *dx*. Phylogenic analysis suggests that *Su*(*dx*) is the *Drosophila* ortholog of mouse Itch. *Su*(*dx*) functions as a negative regulator of Notch signaling and interacts with Notch by direct association of its WW domain 3 (WW3) and a PY motif located on the ICD.

Proteolysis-independent roles of Itch in the positive regulation of the TGF-β signaling. The intracellular signaling downstream ligand-dependent activation of the transforming growth factor-β receptor (TGFβR) is tightly controlled by the ubiquitin machinery.[25] Besides the well acknowledged role of the Smad ubiquitylation regulatory factor (Smurf) E3s in attenuating the TGF-β signaling, Itch-mediated ubiquitylation of Smad2 positively modulates its phosphorylation by activated TGFβR.[11] Under stimulation conditions, Itch facilitates the interaction between the TGFβR and Smad2, and by this means promotes its subsequent phosphorylation in an ubiquitylation-dependent manner.

Among its multiple regulative functions in T-cell homeostasis, TGFβR signaling has a critical role in limiting T-cell-mediated autoimmunity, through the maintenance of peripheral $Foxp3^+CD4^+$ regulatory T_{Reg} cells (reviewed in Rubtsov and Rudensky[26] and references therein). This T_{Reg}-cell population may well play a major role in attenuating T_H2-mediated airway inflammation. Venuprasad *et al.*[27] have recently implicated Itch in TGFβ-mediated modulation of Foxp3 expression and generation of T_{Reg} cells, through the ubiquitin-dependent transcriptional activation of the TGFβ-inducible early gene 1 product (TIEG1). As Foxp3 is a transcriptional target of TIEG1, Itch ablation severely compromises TGFβ-stimulated Foxp3 expression and causes resistance to TGFβ-induced cell growth inhibition. These discoveries have highlighted a novel mechanism by which tch controls allergic responses by influencing the TGFβ signaling.

Cell death regulation

The p53 family members. p73 and p63 share functional similarities with the homologous transcription factor p53, being able to mediate cell cycle arrest and apoptosis in response to DNA damage-induced cellular stress.[28–32] The tumor suppressor activity of p73 and p63 has been further corroborated by the observation that p73 heterozygous mice spontaneously develop a spectrum of histologically different tumors.[33]

Under normal conditions, p73 and p63 protein levels are generally maintained low through Itch-mediated ubiquitylation.[34,35] Both transcription factors are recruited by the WW2 of Itch[8] through a C-terminal PY motif. The PY modules of p73 and p63 span residues 484–487 and 501–504, respectively.[34,35] Like p53, p73 and p63 accumulate in tumor cell lines in response to γ-irradiation or treatment with various chemotherapeutic drugs.[36] In response to genotoxic stress, their induction and transcriptional activation is mainly controlled at the post-translational level, and, at least partially, promoted by DNA damage-caused Itch downregulation.[34,37] The molecular mechanisms responsible for Itch downregulation following cellular stress, and whether or not aberrant expression or regulation of Itch could negatively regulate the tumor suppressive functions of the p53 family members await further investigation.

E2F1-dependent upregulation of p73 mRNA is a key determinant in the mitochondrial pathway of T-cell apoptosis initiated by the TCR signaling.[38–41] Hence, another intriguing scenario wherein Itch may play a critical role in regulating p73 protein stability is activation-induced cell death (AICD) of T lymphocytes. Since, as discussed later in this review, Itch becomes negatively regulated in response to TCR engagement, it is conceivable that stimulation of the receptor would increase p73 protein levels and proapoptotic functions (Figure 3).

While *Itch*$^{-/-}$ $CD4^+$ T cells on the C57BL/6J background exhibit hyperproliferaton following TCR stimulation *in vitro* and expansion of peripheral populations is observed, it is important to consider that the severity of the T_H2 bias is background-dependent.[30] In addition, $CD4^+$ T cells differentiated under type 1 conditions are significantly more susceptible to AICD than T_H0 or T_H2 subsets.[42] Consequently, our postulation of a role for Itch regulation of p73 driven $CD4^+$ T-cell apoptosis *in vivo* may be a key feature of the T_H1 immune response (Figure 3). Indeed, for more complex immune responses, *Itch*$^{-/-}$ $CD4^+$ T cells undergoing T_H1 differentiation may well be preferentially deleted

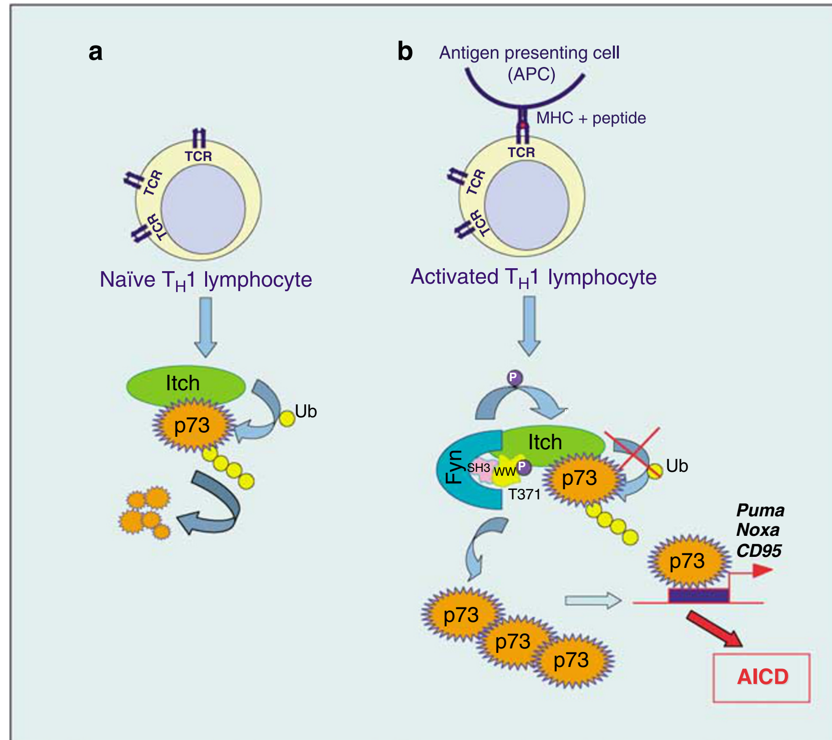

Figure 3 A model for Itch-mediated regulation of activation-induced cell death (AICD). (**a**) In naïve T_H1 lymphocytes, the steady state levels of p73 are kept low by the ubiquitylating activity of Itch. (**b**) After encounter with specific antigens, post-translational p73 upregulation in T_H1 differentiated subsets of lymphocytes could be the result of (i) Itch downregulation, (ii) transient Tyr-phosphorylation of Itch by Fyn, and (iii) the inhibitory competitive action exerted by N4BP1 on Itch substrate recruitment ability. p73 accumulation in T_H1 cells leads to increased transactivation of pro-apoptotic target genes

relative to $CD4^+$ *Itch*$^{-/-}$ cells undertaking expansion under T_H2 differentiation (Figure 1), and could well be a contributing factor to the observation of a $CD4^+$ T cell/T_H2 expansion observed in *Itchy* mice on the C57BL/6J background.[10]

TNFR1 signaling: cellular FLICE-inhibitory protein (c-FLIP$_L$) and receptor interacting protein 1 (RIP1). The involvement of Itch in cell death regulation becomes also evident from its ability to promote tumor necrosis factor-α (TNFα)-induced apoptosis through the proteasomal elimination of the long-splice isoform of the caspase-8 inhibitor, c-FLIP$_L$.[43] The amount of cellular c-FLIP$_L$ crucially determines the extent of caspase-8 recruitment to the adaptor protein FADD and its rate of activation upon binding of TNFα to its type 1 receptor (TNFRI). The specificity for the c-FLIP$_L$ variant is dictated by the selective ability of a cryptic Itch-binding domain to interact with the C-terminal caspase-8-like (CASP) domain, lacking in the shorter isoform. The *in vivo* relevance of these findings is uncovered by the observation that, the *Itchy* mutant mice exhibit protection from fulminant hepatitis, a pathological response to TNFα-receptor activation.

More recently, a role for Itch in negatively regulating inflammatory cytokine signaling by forming a functional complex with TAX1BP1 and A20 has been described. This is mediated by Itch binding to two conserved PY motifs in the zinc fingers of TAX1BP1, an activator of A20. Functionally, this novel signaling complex marks RIP1 for inactivation by proteasomal degradation. Consequently, Itch negatively regulates the amplitude of TNFR1, NF-κB, and JNK signaling initiated by RIP1.[44]

Regulation of epidermal keratinocyte differentiation. Intriguingly, a number of Itch substrates (c-Jun, JunB, p63, Notch, Gli1) are transcription factors controlling epidermal stem cell maintenance and keratinocyte specification, as well as orchestrating the spatiotemporal progression of terminal differentiation.[4,10,35,45] It is therefore likely that Itch-mediated degradation of some or all of these proteins would have a regulative role in skin biology.

A key regulator of the expansion of the basal keratinocyte population, as well as of the epidermal terminal differentiation is p63. According to the current model, the ΔNp63 isoform is responsible for maintaining the basal layer proliferative potential, whereas TAp63 contributes to the stratification and maturation of the suprabasal layers.[46] p63 and Itch colocalize in the adult human normal epidermis, being predominantly distributed in the basal and the upper layer, respectively.[35] The *in vivo* expression gradient of the substrate and the ligase is recapitulated during the *in vitro* differentiation of keratinocytes, when the accumulation of Itch is paralleled by the reduction of ΔNp63.[35] All together, these observations strongly indicate that Itch physiologically controls p63 steady-state protein levels, and, as such, the capacity of the transcription factor to direct the expansion of the basal compartment.[35]

Similarly, Notch receptors have been implicated in governing the balance between proliferative basal cells and terminally differentiating suprabasal epidermal keratinocytes by committing basal progenitors to a spinous cell fate.[47] In normal epidermis, Notch1-3 receptors are primarily expressed suprabasally, where they negatively regulate the transcription of

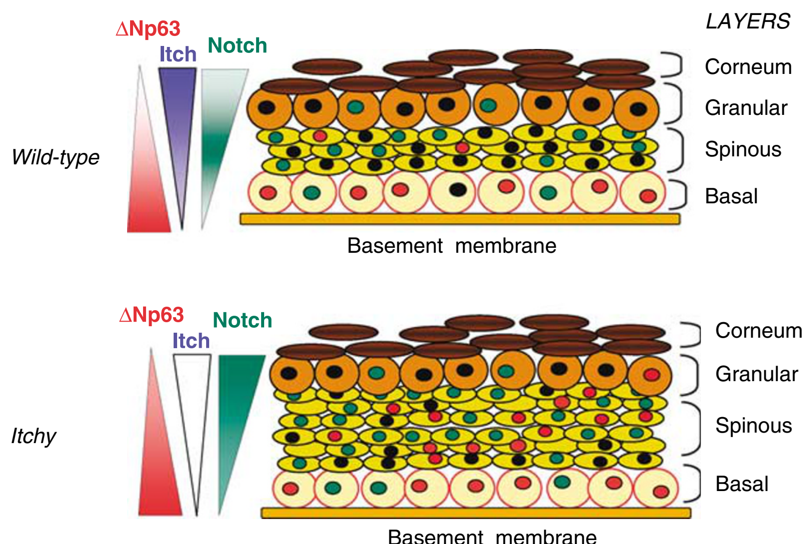

Figure 4 Itch modulates the epidermal keratinocyte differentiation program by targeting multiple substrates for protein ubiquitylation. ΔNp63 (red triangle), the predominant p63 isoform in the epidermis, is exclusively expressed in the basal proliferative compartment, Notch (green triangle) is mainly expressed in the spinous layer, while Itch (blue triangle) is present throughout the epithelium, though it mainly accumulates in the suprabasal cell layers. By regulating both ΔNp63 and Notch protein levels, Itch could exert a role in governing epidermal stratification. Itch would facilitate keratinocytes to exit the basal layer by shortening ΔNp63 half-life in the upper layers and finely tuning Notch expression to promote the basal/spinous transition (upper panel). Lack of Itch would alter the expression of its substrates and lead to epidermal hyperproliferation as a result of an increased number of cells committed to terminally differentiate (lower panel)

genes typically expressed in the basal compartment as well as they activate early genes of the keratinocyte differentiation program. Itch-mediated degradation of Notch would thus contribute to regulate spinous cell fate specification and keratinocyte differentiation (Figure 4).

Abnormal expression of p63 and Notch might be, at least partially, responsible for the increased epidermis thickness phenotype displayed by the *Itchy* mutant mice (Candi and Melino, unpublished observations, Figure 4). Blanpain *et al.*[47] have recently generated a mouse model in which the activated ICD of Notch is constitutively expressed in skin epithelium. Of note, Notch1 ICD transgenic animals display a massive expansion of the spinous layer.

Regulation of receptor trafficking and signaling. A growing body of evidence supports a role for Itch in controlling trafficking of cell signaling receptors at the endosomes. Itch is able to interfere with the endocytic transport at different levels. One such mechanism of action is to direct ligand-activated receptors to the lysosomal degradation pathway.

A recent work by Sundvall *et al.*[48] have highlighted a role for Itch in regulating endocytosis and protein stability of ErbB-4, a receptor belonging to the epidermal growth factor receptor (EGFR)/ERbB family. Unique to the ErbB family, following ligand binding, ErbB-4 is proteolytically cleaved to produce a tyrosine kinase IC fragment, which translocates to the nucleus to regulate transcription. In addition to targeting the ICD of ErbB-4 for lysosome-mediated degradation, Itch may interfere with the ErbB-4 signaling by competing with the WW domain-containing transcriptional coactivator Yes-associated protein (YAP) for binding. Cytoplasmic sequestration of ErbB-4 by Itch would limit the formation of the transcriptionally active ErbB-4/YAP complexes.

Though a direct effect of Itch on the protein turnover of the EGF tyrosine kinase receptor is still a matter of debate, Itch is thought to promote EGFR trafficking and contribute to EGFR signaling downregulation. Itch utilizes its WW domains to recruit the RING finger E3 Cbl, primarily responsible for the degradation of ligand-stimulated EGFR, and, as a result of this cooperative interaction, Itch would control the EGFR kinase activity.[7]

A further evidence supporting a role for Itch in controlling the EGFR signaling pathway is revealed by the discovery that endophilin-A1, a protein involved in clathrin-mediated endocytosis of the EGFR, is a substrate for endosome-localized Itch.[9] Lacking the canonical PY recognition motif, endophilin-A1 is an additional atypical Itch protein interactor. The unusual nature of this association is achieved through the SH3 domain of endophilin-A1 and a consensus-binding motif (PXRPPXPR) within the PRR region of Itch. Upon EGFR stimulation, endophilin-A1 and the receptor translocate to the endosomal compartment. In addition, EGFR activation stimulates Itch-mediated endophilin-A1 ubiquitylation, though the precise functional role of this modification in protein trafficking regulation awaits further investigation.

In view of the interference of Itch with the EGFR signaling pathway, it is attractive to speculate that the hyperproliferation of epidermis and stomach epithelium manifested by the *Itchy* mice[1] (Candi and Melino, unpublished observations) could also be the result of increased EGFR signaling.

Itch has been also implicated in the agonist-dependent ubiquitylation of the chemokine receptor CXCR4 at the plasma membrane. Ubiquitylated CXCR4 is then targeted for lysosomal degradation. In addition, Itch regulates endosomal sorting of activated CXCR4. Upon receptor internalization, ubiquitylated CXCR4, and Itch traffic to endosomes. In this compartment, Itch mediates the ubiquitylation of Hsr, a protein of the endosomal sorting machinery, which controls ubiquitin-dependent trafficking

of cargo, including CXCR4, to the degradative pathway.[5] It has been speculated that conjugation of ubiquitin to Hsr would affect its ability to sort cargo. Hence, Itch-mediated ubiquitylation events would, in turn, promote CXCR4 targeting for lysosomal destruction.

A different mechanism of regulation of the endosomal trafficking by Itch is accomplished by controlling endocytosis and hence the abundance of ion channels at the plasma membrane. Itch-mediated multiubiquitylation of members of the transient receptor potential (TRP) family of cation channels serves as an internalization signal, resulting in downregulation of their cell surface expression and basal activity.[6] However, once TRP channels undergo ubiquitin conjugation, endocytosed proteins are not degraded, but rather they recycle back to the plasma membrane.

Regulatory Mechanisms Governing Itch Catalytic Activity and Substrate Specificity

Itch catalytic activity and substrate recognition properties are subjected to many levels of regulation, including several post-translational modifications and interaction with adaptor proteins.

Phosphorylation. Phosphorylation of protein substrates, which facilitates their recognition by the E3s, represents a common regulatory pathway of the ubiquitin conjugation process. Unlike the RING finger-containing E3s, the HECT type ligases typically recognize protein substrates independently of their phosphorylation state. Phosphorylation of the E3 enzymes themselves has been recently emerged as a critical post-translational modification modulating their enzymatic activity or substrate recognition properties. Biochemical and genetic studies have shown that a crucial regulatory mechanism of Itch catalytic activation is Jun N-terminal Kinase1 (JNK1)-mediated Ser/Thr phosphorylation of the E3 upon TCR engagement.[49] The JNK1 phosphoacceptor sites of Itch have been mapped within the PRR region, where three pro-directed Ser/Thr residues are located.[50] In response to T-cell activation, JNK1 docking to the D domain, located in the proximal region of the HECT domain (residues 595–604), allows multiple phosphorylation of the E3 on residues Ser199, Thr222, and Ser232. In its unphosphorylated state, Itch enzymatic activity is negatively regulated through intramolecular interactions between the central region, including the WW and PRR motifs, and the C-terminal HECT domain. Following phosphorylation, Itch undergoes a conformational change, which destabilizes the self-inhibitory intramolecular interactions, present within the ligase, thus allowing substrate recruitment and catalytic activation.[50]

Key to the catalytic activity of HECT E3 ligases is the conformational flexibility conferred by a hinge-like region within the bi-lobed HECT structure.[51] This flexible hinge region is critical for juxtaposing the catalytic cysteine residues of the E2 and the HECT domain, during ubiquitin transfer. Most likely the Itch auto-inhibitory conformation, whereby the PRR and HECT directly interact, prevents such flexibility within the HECT domain, greatly reducing its catalytic activity.

The activation of the JNK1 signaling pathway decreases protein turnover of the Jun family members, thus attenuating the polarization of $CD4^+$ T lymphocytes into T_H2 effector cells (Figure 1). JNK1 can also signal c-$FLIP_L$ protein ubiquitylation via a similar phosphorylation-dependent activation of Itch.[43]

These biochemical findings are genetically supported by the phenotypic similarities in $CD4^+$ T cells between JNK1 null, mitogen and extracellular kinase kinase 1 kinase-deficient $(Mekk1)^{\Delta KD}$ and *Itchy* mice.[10,49,52] The $Mekk1^{\Delta KD}$ mice express an inactive form of MEKK1, an *in vivo* upstream activator of JNK, ERK and p38 MAPK signaling.[53] Hampering Itch-mediated degradation of Jun family members, achieved either through Itch mutational inactivation or as a result of JNK1 chemical or peptide inhibition, enhances the expression of T_H2 cytokine genes (IL-4, 5, 10 and 13) in $CD4^+$ T cells challenged with T_H2 polarization conditions.[49] Hence, the MEKK1-JNK signaling pathway plays a crucial role in the negative regulation of T_H2 differentiation as well as of peripheral immune tolerance induction.[18,49]

In contrast to the positive regulation induced by the JNK1 pathway, the Src kinase, Fyn, phosphorylates Itch on Tyr 371 residue, leading to the inhibition of its E3 activity.[54] Transient Tyr phosphorylation of Itch occurs in T lymphocytes in response to TCR engagement (Figure 1). Fyn binding to Itch is mediated through the interaction of Fyn SH3 domain with WW3 of Itch. Similarly, Tyr371 is located at the N-terminus of Itch WW3 domain. Following Tyr371 phosphorylation, the association between Itch and its substrates, such as JunB is hampered. Hence, after TCR stimulation, Itch-mediated ubiquitin conjugation to JunB is reduced and the transcriptional factor stabilized. Due to augmented JunB degradation, Fyn-deficient T lymphocytes display a decreased production of T_H2 cytokines. An additional level of regulation of Fyn activation in response to TCR stimulation could be achieved through the stabilization of p73. In activated T_H1 cells, the negative control exerted on Itch may result in increased protein turnover rate and induction of AICD (Figure 3).

The MEKK1-JNK1 and Fyn signaling pathways oppositely regulate the turnover of Jun family members and the immune response by counteracting Ser/Thr and Tyr phosphorylation. These dynamic phosphorylation events, in turn, would result in the attenuation and potentiation of T_H2 differentiation, respectively (Figure 1).

Ubiquitylation against deubiquitylation. Itch undergoes auto-ubiquitylation *in vivo*.[8,49,50,55] The molecular nature and the physiological relevance of Itch self-modifications have just begun to be elucidated. It has been recently proposed that, similarly to other E3s, Itch autocatalytic activity negatively controls its protein stability.[55] Nevertheless, Itch auto-ubiquitylation can also provide a non-proteolytic regulatory function, such as modulating its cellular localization or catalytic activity[49,50] (Scialpi, Melino, Bernassola, personal communication). Ubiquitin self-conjugation of Itch is subjected to different levels of control, being stimulated by JNK activation[50] or inhibited by the association with the protein interactor, Nedd4-binding partner-1 (N4BP1),[8] illustrated later in this review.

Furthermore, Itch is a substrate for the deubiquitylating activity of FAM/USP9X, a member of the ubiquitin-specific proteases (USPs).[55] By counteracting Itch self-ubiquitylation, FAM/USP9X protects the ligase from proteasomal

degradation. It could be also possible that FAM/USP9X serves to generate mono-ubiquitylated species, thus adding an additional level of control on the E3.

Interaction with adaptor proteins. The HECT E3s can cooperate with accessory and adaptor proteins, which contribute to modulate their substrate recruiting capacity, subcellular localization, and enzymatic activity (reviewed in Shearwin-Whyatt [56] and references therein). Itch-interacting proteins enable further specificity to the ubiquitylation reaction. Furthermore, the existence of noncanonical substrates[4–6] suggests that Itch could be recruited to some of its protein targets through an adaptor molecule.

N4BP1 is a novel Itch negative regulator, acting as a competitor of its substrate recruitment ability.[8] Although N4BP1 does not contain canonical WW domain docking sites, its binding to these modules determines displacement of the substrates from the ligase. Itch-mediated transfer of ubiquitin to its protein targets is then prevented, and the half-life of the substrates prolonged. As a result, N4BP1 potentiates the transcriptional activity of both p73 and c-Jun. The competition mechanism implies that the selectivity of Itch for the substrates could be modulated either by changes in the affinity of binding to the adaptor molecule, or by alterations of their cellular availability. In this scenario, TCR stimulation-induced accumulation of c-Jun and p73 could be achieved either through N4BP1 induction, or through chemical/conformational modifications, which would enhance the affinity of N4BP1 for the E3.

Nedd4 family interacting protein-1 (Ndfip1) is a membrane-associated protein, originally discovered in an interaction screening using the WW domains of mouse Nedd4.[57] Ndfip1 possesses two N-terminal PY motifs, which mediate direct interaction with Nedd4 as well as Itch.[58] In yeast, the Ndfip1 ortholog Bsd2 functions as an adaptor, recruiting the HECT domain E3 Rsp5 to substrates, thus enhancing the recognition and removal of misfolded membrane proteins.[59] In mammals, the biological outcome of such interaction is promoting itch-mediated degradation of target substrates.[60] This physical association becomes particularly relevant after T-cell stimulation, when, as a result of Ndfip1 induction, Itch is relocalized from the intracellular vesicles to the inner surface of the plasma membrane. Consequently, enhancement of JunB degradation prevents T_H2 cytokine production. This functional interaction is corroborated by the phenotype manifested by the *Ndfip1* knockout mice. Similarly to the *Itchy* mice, *Ndfip1* deficient animals are prone to develop a severe skin and lung inflammation, accompanied by hepatomegaly and splenomegaly, and high numbers of infiltrating eosinophils at the inflammatory sites. Lack of *Ndfip1* predisposes T lymphocytes toward a T_H2 phenotype, and biases them toward the production of T_H2 cytokines.

Another Itch protein interactor exhibiting a regulatory role is the vertebrate homolog of *Drosophila* Numb.[21] The mammalian Numb protein, whose phosphotyrosine-binding (PTB) domain recruits the C-terminal ICD of Notch, cooperatively enhances Itch-catalyzed ubiquitylation of the membrane-bound receptor, and specifically promotes the ICD degradation following receptor activation (Figure 2). This effect is achieved through direct binding of Numb to Itch WW1/2 domains. By promoting rapid degradation of Notch1 ICD, Numb prevents the translocation of the activated receptor to the nucleus and, ultimately, inhibits Notch-dependent signal transduction.

An additional Itch substrate whose modification by ubiquitin requires the accessory role of Numb is the transcription factor Gli, which mediates the effect of Hedgehog signaling in neural stem cell maintenance and self-renewal.[61] Numb stabilizes the ligase:substrate complex, and functionally synergizes with the ligase in targeting Gli for ubiquitin-dependent proteasomal proteolysis. Since accumulation of Gli in the nucleus is the major mechanism regulating its transcriptional activation, its lost from the nuclear compartment suppresses the expression of Hedgehog target genes. As such, Numb acts as an antagonistic regulator of Hedgehog signaling during cerebellar development.

The C-terminal WW domains (2–4) of the HECT type E3s are known to directly mediate the association with the substrate providing high affinity binding to the PY motifs.[8,62,63] A regulatory function has been instead ascribed to the first WW domain. As an example, WW1 of Nedd4 interacts with cofactors that regulate ubiquitin/proteasome-dependent proteolysis of bound substrates. In principle, by interacting with regulatory WW domains of Itch, Numb might act as an adaptor facilitating or stabilizing the interaction between Itch and its substrates.

Conclusion

The ubiquitin ligase Itch plays key roles in different cellular contexts, in virtue of its functionally distinct substrates. Nevertheless, Itch targets can be simplistically categorized in two main classes: transcription factors and growth factor receptors, some of them acting as gene expression regulators as well. Aberrant accumulation of several signaling proteins such as the Jun family members and Notch due to the loss of Itch protein, critically contributes to the autoimmune phenotype of the *Itchy* mice. A significant number of the transcriptional regulators targeted by Itch for proteasomal destruction are crucially involved in controlling cell growth, differentiation, and apoptotic processes. This raises the intriguing possibility that their inappropriate removal, due to altered Itch regulation, signaling or activity, would be tightly linked to malignant transformation and chemoresistance.

Acknowledgements. We sincerely apologize for not being able to cite some relevant references because of space constraints. This work has been supported by EU-Grant EPISTEM LSHB-CT-019067; the AIRC Grant 1338; the ISS Grant no. 530/F-A19, Grant Telethon GGPO4110, and Philip Morris grant awarded to GM, and by the EU NeOEs Rubicon and NeuroNE, the Israel Science Foundation, the German Israeli Foundation, the Israel Cancer Research Fund, and the Dr. Miriam and Sheldon Adelson Medical Research Foundation awarded to AC.

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