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Itch self-polyubiquitylation occurs through lysine-63 linkages

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ABSTRACT

Itch, an E3 protein ubiquitin ligase (E3), which belongs to the homologous to E6-AP carboxy terminus (HECT)-type subfamily, catalyzes its own ubiquitylation. The precise nature of Itch-mediated self-modification and its biological outcome are not completely understood. Here, we show that Itch auto-ubiquitylation is an intermolecular reaction generating Lys63-linkages, rather than the Lys48-linked polyubiquitin chains that target proteins for proteasomal degradation. As a result, Itch is a relatively high stable protein, whose levels are not significantly affected by treatment by either proteasome or lysosome inhibitors. Furthermore, we demonstrate that the decay rate of a catalytic inactive Itch mutant, which is devoided of self-ubiquitylating activity, is barely indistinguishable from the one of the wild-type protein. These data definitely establish a nondegradative role for Lys63-linked Itch self-ubiquitylation.

1. Introduction

Itch is an E3 ubiquitin ligase (E3) belonging to the homologous to E6-AP carboxy terminus (HECT)-type E3 subfamily. Similarly to the other members of this family, Itch is composed of three distinct domains: an N-terminal C2 domain, followed by four protein-interacting WW domains, and a C-terminal catalytic HECT domain. The C2 domain, which is about 116 amino acid residues, is thought to play a role in membrane targeting and subcellular localization. The WW module is a short domain of approximately 40 amino acids that mediates the interaction with substrates, generally containing Pro-rich, PPXY, or phospho-Ser/Thr motifs. The HECT domain is a large module, which recruits the E2 conjugating enzyme (E2) and provides the intrinsic enzymatic E3 activity [1]. Upon recruitment of the E2, the catalytic Cys residing at the C-terminus of HECT domain accepts ubiquitin from the E2, through the formation of a ubiquitin-thioester intermediate. Consequently, the

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Abbreviations: c-FLIP$_L$, cellular FLICE-inhibitory protein; CHX, cycloheximide; E1, E1 ubiquitin activating enzyme; E2, E2 conjugating enzyme; E3, E3 protein ubiquitin ligase; HECT, homologous to E6-AP carboxy terminus; MS, mass spectrometry; Met-Ub, methylated ubiquitin; MEFs, mouse embryonic fibroblasts.

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ubiquitin molecule is conjugated to Lys residues of the target protein, through the formation of isopeptide linkages involving the C-terminal Gly carboxyl group of ubiquitin [2–5].

Through ubiquitin-dependent proteolysis of target proteins, Itch controls distinct biological processes, including T cell differentiation, immune responses, cell death and skin morphogenesis (reviewed in [6,7]). In particular, Itch E3 activity is implicated in the regulation of the apoptotic pathway through its ability of promoting the ubiquitylation and the subsequent proteasome-dependent degradation of the p53 family member, p73 [8], and the cellular FLICE-inhibitory protein (c-FLIP$_L$) [9]. Itch ubiquitin ligase activity is not only implicated in catalyzing the elongation of poly-ubiquitin chains, but it also regulates the stability of transmembrane receptors through canonical mono- or multi-ubiquitylation [10,11]. Proteolysis-independent ubiquitylation events have also been ascribed to the E3 activity of Itch [12–14].

In addition to catalyzing conjugation of ubiquitin to specific substrates [8,9,15,16], and similarly to other HECT E3s [17,18], Itch promotes its own ubiquitylation [16,19–21]. Although Itch auto-ubiquitylation has been demonstrated in cells [16], 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 auto-catalytic activity may negatively control its protein stability [20]. Itch auto-ubiquitylation is inhibited by the ubiquitin specific protease USP9X, which appears to influence Itch protein levels by counteracting self-ubiquitin conjugation [20].

The auto-ubiquitylating activity is thought to mainly act as a regulatory mechanism that controls the abundance of E3s by marking them for degradation [22,23]. However, nonproteolytic functions have recently been ascribed to E3 self-ubiquitylation [18,24]. The fate of ubiquitylated proteins is determined by the nature of ubiquitin attachment (e.g. mono- *vs*. poly-ubiquitylation) and by the type of isopeptide linkage forming the polyubiquitin chain. All seven internal residues of ubiquitin can potentially act as chain-elongation sites [25]. Lys48-linked poly ubiquitin chains label proteins for 26S proteasome-mediated proteolysis, while ubiquitin chains formed through Lys63, or other linkages, typically act as nondegradative signals [17,26–31]. In this study, we analyze the molecular mechanisms of Itch self-ubiquitylation and investigate its possible involvement in the regulation of Itch protein stability.

2. Materials and methods

2.1. Reagents and antibodies

The following reagents were used: cycloheximide (CHX, Sigma, St. Louis, MO, USA), monensin (Sigma), NH_4Cl (Sigma), chloroquine (Sigma), MG132 (Calbiochem, San Diego, CA, USA), N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES, Sigma), Adenosine 5′-triphosphate (Roche Molecular Biochemicals, Mannheim, Germany), ubiquitin aldehyde (Sigma). The E1 ubiquitin activating enzyme (E1), the E2 UbcH7, and recombinant purified wild-type (WT) and mutant ubiquitins were from Boston Biochem (Cambridge, MA, USA). The ubiquitin mutants were either molecules in which all Lys residues were changed to Arg (K0-Ub), or in which all Lys residues but one were replaced by Arg (K63-Ub), or in which individual Lys residues were mutated to Arg (K48R-Ub, K63R-Ub).

The following antibodies were used: mouse monoclonal anti-Itch (BD Biosciences Pharmingen, CA, USA), mouse monoclonal anti-Myc (Cell Signaling Technology Inc., Beverly, MA, USA), mouse monoclonal anti-p21 (Sigma), mouse monoclonal anti-β-actin (Sigma), rabbit polyclonal anti-Flag (Sigma), and mouse monoclonal anti-ubiquitin (Zymed, San Francisco, CA, USA).

For Itch expression in bacterial cells, we employed a modified construct (GST-ΔC2-Itch) lacking the N-terminal region to avoid solubility problems due to the presence of the C2 domain. ΔC2-Itch retains direct substrate interaction abilities as well as a functional catalytic domain. On the contrary, all plasmids utilized for the expression of wild-type and C830A-Itch in eukaryotic cells encode the full-length proteins [8].

2.2. Cell culture and transfection conditions

Human embryonic kidney HEK293T (ATTC, Manassas, VA, USA) and *Itch*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin and 10% FBS (Gibco). Transfections of HEK293T cells and MEFs were performed using the calcium phosphate method, and the Effectene reagent (Qiagen, Santa Clara, CA, USA), respectively.

2.3. Measurement of Itch half-life

Itch turnover rate was determined using CHX inhibition of protein synthesis. *Itch*$^{-/-}$ MEFs were transiently transfected with either wild-type or C830A mutant Itch. Twenty-four hours after transfection CHX was added to the culture media to a final concentration of 80 μg/ml. Cells were harvested at indicated time points, and equal amounts of cell lysates were subjected to SDS-PAGE and analyzed by immunoblot (IB). The same protocol was used to analyze the half-life of endogenous Itch in HEK293T cells.

2.4. IB analysis and immunoprecipitation

To prepare whole cell extracts for IB analysis, cells were lysed in Triton lysis buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 50 mM NaF, 1 mM EDTA 1 pH 8, 0.1% Triton), supplemented with proteases and phosphatase inhibitors. Proteins were separated by SDS/PAGE and blotted onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with PBS-T (Phosphate-buffered saline and 0.1%Tween-20)/5% nonfat dry milk for 1 h, and then incubated with primary antibodies for 2 h at RT. After washing, membranes were incubated for 1 h at RT by using the appropriate horseradish peroxidase-conjugated secondary antibody (PerkinElmer, Waltham, MS, USA). Detection was performed with the ECL Western Blot Chemiluminescence Reagent (PerkinElmer). For

immunoprecipitation, cells lysates were precleared with protein A/G-Sepharose beads (GE Healthcare, UK) for 3 h, and then immunoprecipitated with 1 μg per sample of the appropriate antibodies, previously adsorbed on protein A/G-Sepharose beads (Roche Molecular Biochemicals). Immunocomplexes were washed six times in Triton lysis buffer and then used for *in vitro* ubiquitylation assay or eluted by boiling in SDS loading buffer.

2.5. *In vitro ubiquitylation assays*

The *in vitro* self-ubiquitylation assay was carried out as previously described [8]. Briefly, the ubiquitylation reaction mixture contained 25 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, 2.5 mM ATP, 4 mM $MgCl_2$, 2 μl of bacterial purified wheat E1, 0.1 μg of a human UbcH7, 100 ng of bacterially purified recombinant wild-type or C830A mutant GST-ΔC2-Itch, and 5 μg of Flag-tagged ubiquitin. After incubation for 90 min at 30 °C, the reactions were terminated by adding SDS loading buffer, resolved by SDS-PAGE, followed by IB with anti-ubiquitin or anti-Itch antibodies.

Alternatively, the *in vitro* ubiquitylation reaction was performed utilizing Flag-Itch immunopurified from HEK393T cells after transient transfection. Briefly, Itch immunocomplexes were washed twice with equilibration buffer (25 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.01% Triton and 10% glycerol). For each sample, 30 ng of E1, 0.5 mg of UbcH7 and 5 μg of ubiquitin were added to the ubiquitylation reaction mixture (2.5 mM Tris–HCl, 0.7 mM DTT, 4 mM ATP, 10 mM $MgCl_2$, 0.1 mM ubiquitin aldehyde). After incubation at 30 °C for the indicated time points, self-ubiquitylated Itch was eluted from the beads by boiling in SDS loading buffer, resolved by SDS/PAGE, followed by IB anti-Itch.

3. Results

3.1. *Itch self-polyubiquitylation occurs through an intermolecular mechanism*

Consistent with previous reports [8,16,19,20], Itch acts in concert with the E2 UbcH7 to promote its own ubiquitylation in a cell-free system (Fig. 1A, lanes 2–4). To further explore the molecular mechanism of Itch auto-modification, we utilized a mutant Itch in which the highly critical Cys residue in its HECT domain was mutated to Ala (Itch-C830A) [8]. The C830A mutation abrogates Itch ligase activity, and consequently, its auto-ubiquitylating capacity [21]. To assess whether Itch-mediated auto-ubiquitylation occurs through an *in cis* or *in trans* reaction, we tested the ability of Itch-C830A to serve as a substrate for the wild-type enzyme in an *in vitro* ubiquitylation assay. Interestingly, mutated Itch was properly poly-ubiquitylated by the wild-type E3, demonstrating that Itch can catalyze the transfer of ubiquitin from its catalytic Cys to a second Itch molecule which serves as a substrate (Fig. 1A, lane 2). These results are in line with our previous observation that the catalytic inactive mutant is, at least moderately, ubiquitylated *in vivo*, likely due to the activity of endogenous Itch [21].

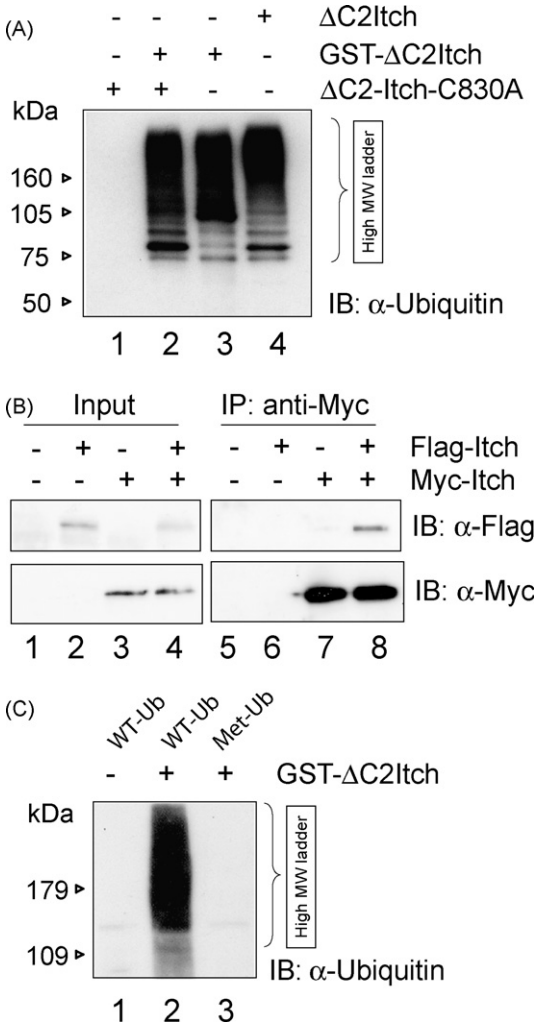

Fig. 1 – Itch self-ubiquitylation acts through an *in trans* mechanism. (A) The Itch-C830A catalytic inactive Itch mutant serves as a substrate for the ubiquitylation activity of wild-type Itch. The *in vitro* ubiquitylation reaction was performed using bacterially purified ΔC2-Itch-C830A (about 70 kDa) as a substrate for wild-type GST-ΔC2-Itch (about 100 kDa). The polyubiquitylation ladders of ΔC2Itch-C830 and GST-ΔC2Itch start at approximately 75 and 105 kDa, respectively, indicating that the self-ubiquitylation reaction involves two separate Itch molecules. (B) HEK293T cells were co-transfected using the calcium-phosphate method with flag-tagged- and myc-tagged-Itch. Forty-eight hour after transfection, cell lysates were immunoprecipitated with monoclonal anti-myc antibody and IB analysis was performed by using either anti-flag or anti-myc antibodies. Inputs and immunoprecipitates are shown on the left and right panel, respectively. (C) Bacterially purified GST-ΔC2Itch was subjected to an *in vitro* ubiquitylation reaction in the presence of either wild-type (lane 2) or Met-Ub (lane 3) that can only support mono-ubiquitylation. Ubiquitylated Itch was detected by IB using anti-ubiquitin antibody.

To validate the intermolecular mechanism of Itch auto-catalysis, we performed *in vivo* pull-down assays using differently tagged-Itch molecules as protein interactors. Reciprocal binding assays revealed that Itch is indeed able to establish intermolecular interactions (Fig. 1B, lane 8, and data not shown).

To assess the nature of Itch-catalyzed self-ubiquitylation, we utilized methylated ubiquitin (Met-Ub), whose modification prevents the elongation of polyubiquitin chains, in a cell-free conjugation assay. The high molecular weight ubiquitin conjugates formed through the incorporation of wild-type ubiquitin were completely abolished in the presence of Met-Ub (Fig. 1C, compare lane 2 and 3), thus demonstrating that Itch is self-modified by polyubiquitylation.

3.2. *Itch generates self-assembled polyubiquitin chains that require internal Lys residue 63 of ubiquitin*

We next sought to determine the type of self-polyubiquitin chains generated by Itch. Utilizing an *in vitro* ubiquitylation assay, we found that immunopurified Itch is still able to catalyze its own ubiquitylation in the presence of a ubiquitin mutant lacking Lys48 (K48R-Ub) (Fig. 2A, lane 3). Similarly to a ubiquitin mutant lacking all Lys residues (K0-Ub), substitution of Lys63 with Arg (K63R-Ub) failed to support Itch auto-ubiquitylation (Fig. 2A, compare lanes 4 and 6). On the contrary, recombinant ubiquitin lacking all Lys residues but Lys63 (K63-Ub) was properly incorporated into self-polyubiquitin chains (Fig. 2A, lane 5). The relative increase in ubiquitin conjugates observed in the presence of K63-Ub (lane 5) could be explained by assuming that under *in vitro* conditions, Lys residues other than Lys63 could be partially utilized for polyubiquitin chain formation. These results indicate that Itch auto-ubiquitylation occurs through Lys63-linked polyubiquitin.

To rule out the possibility that Lys63-linkages were catalyzed by a different E3 activity co-immunoprecipitating with Itch, we tested the ability of bacterially purified Itch to promote its own ubiquitylation in the presence of the aforementioned ubiquitin mutants. We indeed observed that the formation of polyubiquitin chains was abolished only by using the K63R-Ub mutant (Fig. 2B, lane 8), while Itch self-ubiquitylation was retained in the presence of K48R-Ub (Fig. 2B, lane 7).

Mass spectrometry (MS) analysis of the self-polyubiquitin chains generated in an *in vitro* ubiquitylation assay confirmed that Lys63 of ubiquitin was the most abundant ubiquitylated species (Alessi and Zagorska, personal communication). All together, these findings demonstrate that Itch catalyzes the generation of self-ubiquitin chains that involve Lys63 of ubiquitin.

3.3. *Itch is a stable protein*

The inability of Itch to synthesize its own K48-based polyubiquitin chains, which is the signal for proteasome recognition, prompted us to analyze the half-life of endogenous Itch. We found that Itch is a highly stable polypeptide, whose decay rate is not appreciable up to 8 h of protein synthesis blockade (Fig. 3A). On the contrary, p21 protein levels declined 1 h after treatment (Fig. 3A). Furthermore, the steady state levels of endogenous Itch were not altered by either incubation with the proteasome inhibitor MG132, or following exposure to a panel of inhibitors of lysosomal hydrolases (Fig. 3B). Collectively, these findings indicate that endogenous Itch degradation is a relatively slow process. Under our conditions, Itch protein levels are not significantly regulated by either proteasome-dependent degradation or by lysosomal activities, and are unlikely governed by self-ubiquitylation occurring *in vivo*.

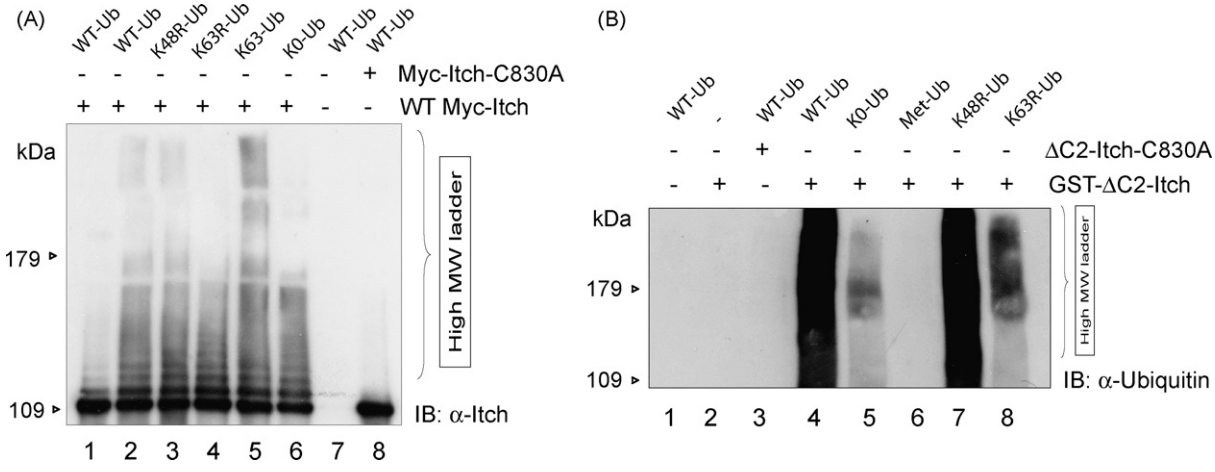

Fig. 2 – Itch self-polyubiquitylation involves Lys63 linkages. (A) HEK293T cells were transfected with plasmids coding wild-type and C830A catalytic inactive Itch. After cell lysis, Itch was immunoprecipitated with anti-myc antibody, followed by an *in vitro* ubiquitylation assay. The ubiquitylation reaction was carried out using WT (lanes 1, 2, 7 and 8), K48R (lane 3), K63R (lane 4), K63 (lane 5), or K0 (lane 6) ubiquitin mutants for 90 min at 30 °C. As a negative control, the ubiquitylation reaction was stopped prior incubation at 30 °C (lane 1). Itch ubiquitylated forms were detected by IB using anti-Itch antibody. (B) Bacterially purified GST wild-type and C830A ΔC2Itch fusion proteins were subjected to an *in vitro* ubiquitylation assay in the presence of WT-Ub (lanes 1, 3, and 4), K0-Ub (lane 5), Met-Ub (lane 6), K48R-Ub (lane 7), or K63R-Ub (lane 8). The assay was analyzed by IB using anti-ubiquitin antibody.

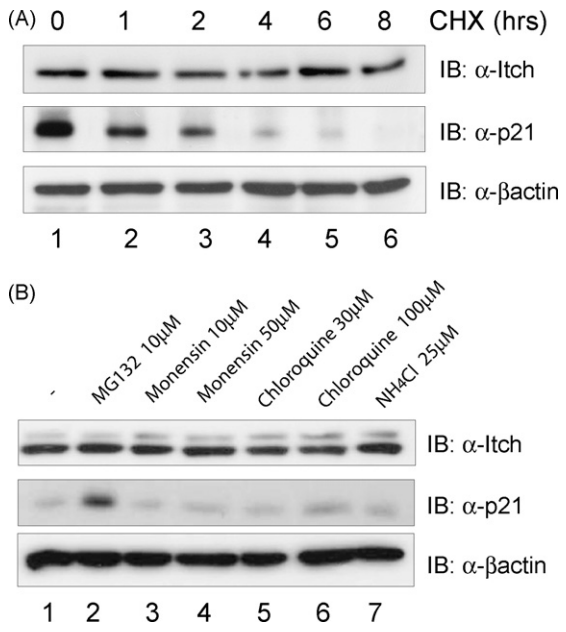

Fig. 3 – The steady state degradation of Itch is a relatively slow process. (A) Determination of endogenous Itch protein half-life. HEK293T were treated with CHX (80 μg/ml) for the indicated time points. Cell lysates were analyzed by IB using anti-Itch, anti-p21 (as a positive control for protein synthesis blockade) and anti-β-actin antibodies. (B) Effect of proteasome and lysosomal inhibitors on the steady-state level of endogenous Itch. HEK293T cells were treated with proteasomal (MG132, lane 2) and lysosomal hydrolases (monensin, chloroquine and NH_4Cl, lanes 3–7) inhibitors at the indicated concentrations for 6 h. Endogenous Itch steady-state levels were determined by IB with anti-Itch antibody. Protein levels of the cell cycle inhibitor p21 were assessed as a positive control for proteasomal degradation.

3.4. *Itch self-ubiquitylating activity of is not required for its degradation*

To further rule out the involvement of Itch auto-ubiquitylating activity in the regulation of its degradation, we reconstituted Itch deficient MEFs with either wild-type or C830A mutant Itch, and their steady-state levels were monitored in the absence or in the presence of the proteasome inhibitor MG132. As shown in Fig. 4A, the two proteins display similar expression levels, and were equally insensitive to proteasome inhibition. As an internal control, we measured p21 and c-Jun induction in response to MG132 treatment. This result confirms that lack of enzymatic activity does not affect Itch protein stabilization. A comparison of their decay rate revealed that wild-type and C830A Itch are both highly stable polypeptides (Fig. 4B).

4. Discussion

Though Itch auto-ubiquitylation has been previously reported, we have further characterized the molecular mechanisms underlying this post-translational modification. In the present study, we provide evidences that Itch engages an intermolecular reaction generating Lys63 polyubiquitin chains, and that this auto-modification does not regulate Itch protein stability.

Consistently with the nonproteolytic self-ubiquitylating activity of Itch, the catalytic inactive C830A mutant was as stable as the wild-type counterpart, and displayed similar sensitivity to the proteasome inhibitor treatment. All together, these findings definitely prove that, in contrast to other E3s [22,23], the self-polyubiquitin chains generated by Itch do not serve as either proteasome or lysosome targeting.

The apparent discrepancy of our conclusions with the stabilizing activity manifested by the deubiquitylating enzyme USP9X on Itch protein levels [20], may be explained by assuming that Itch may serve as a substrate for other not yet discovered E3s, and that the ubiquitin protease would utilize alternative non-self-catalyzed ubiquitin chains as a substrate. In support of this notion, USP9X displays specificity for polyubiquitin chains formed through different linkages, including Lys63 [31], Lys29 and Lys33 [30], and possibly Lys48 [32,33] bonds.

The analysis of the primary structure of Itch reveals that different lysine residues are spread along the entire protein. In an effort to determine the ubiquitin Lys acceptor sites targeted for protein auto-ubiquitylation through a MS approach, six self-modified Lys residues have been identified (Alessi and Zagorska, personal communication). The analysis of Itch mutants, in which those Lys have been replaced to Arg, did not show any significant difference in their self-ubiquitylating capacity as compared to the wild-type protein. This result might likely reflect the fact that the extent of protein coverage by MS was not complete. Moreover, the inability of Itch to catalyze discrete mono-ubiquitylated bands in the presence of Met-Ub and the K0-Ub mutant, confirms that different Lys residues of Itch can be utilized as acceptor sites for ubiquitin transfer. Without the knowledge of the specific Lys residues acting as polyubiquitin chains anchors, there is an open question remaining about the possible role(s) of Itch self-modification.

Though HECT-type E3s have been considered to be constitutively active and regulated only at the level of target binding, there is emerging evidence showing that they are finely controlled either by phosphorylation [16,34] or through the association with adaptor proteins [35,21]. It is time to speculate that auto-ubiquitylation could serve as a further regulatory mechanism, which may affect Itch function at different levels. One possibility would be direct interference with its catalytic activity. Alternatively, self-ubiquitylation could induce a conformational change influencing the post-translational modification pattern of Itch. For instance, upon ubiquitylation, Itch phosphorylation state could be modified, hence allowing an alteration of the inhibitory intramolecular interactions regulating its catalytic activation [19]. As a result of both molecular mechanisms, Itch capacity to conjugate ubiquitin to protein targets would be affected. A similar outcome for self-ubiquitylation has been described by Ben-Saadon et al. [24], reporting that the ability of the RING-finger E3 Ring1B to monoubiquitylate histone H2A is increased upon generation of self-atypical Lys6, Lys27 and Lys48 mixed ubiquitin chains. In addition, Lys63-linked auto-ubiquitylation

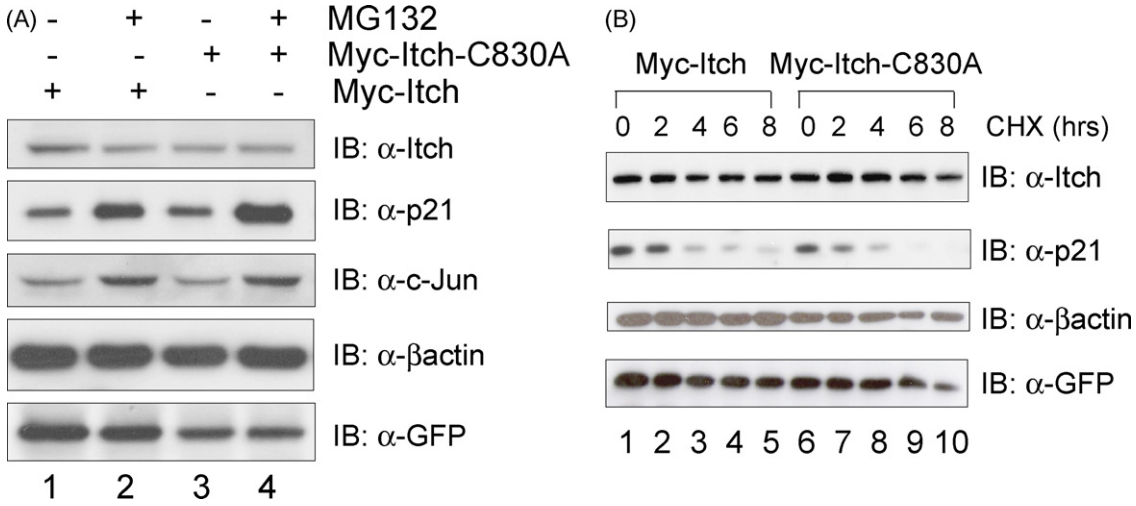

Fig. 4 – Lack of self-ubiquitylating activity does not affect Itch protein stability. (A) Effect of the proteasome inhibition on the steady-state levels of wild-type and C830A catalytic inactive Itch. *Itch*$^{-/-}$ MEFs were co-transfected with GFP along with either wild-type or C830A catalytic inactive mutant myc-Itch. Twenty-four hours after transfection, cells were treated with 20 μM MG132 for 5 h, harvested and analyzed by IB by using anti-Itch antibody. GFP proteins levels were used to normalize transfection efficiency. (B) Protein decay rates of wild-type and C830A mutant Itch. *Itch*$^{-/-}$ MEFs were transfected as indicated in panel A. Twenty-four hours after transfection, cells were treated with 80 μg/ml CHX for the indicated times. Cell lysates have been examined by IB using anti-Itch antibody.

of TRAF6 is required for its ability to ubiquitylate NEMO, and for subsequent IKK and NF-κB activation [36].

Both mono- and poly-ubiquitylation of protein substrates have been associated with internalization, sorting and changes in their subcellular localization [27,37,38]. Hence, ubiquitin conjugation may represent a signal for Itch to translocate to distinct cellular compartments, which ultimately, would modify its accessibility to certain substrate molecules. Itch is predominantly localized to early and late endosomal compartments and lysosomes, though a small fraction displays a perinuclear and nuclear distribution [11,20]. Numerous Itch substrates are transcription factors mainly residing in the nuclear compartment. Hence, self-ubiquitylation may represent an auto-regulatory mechanism controlling Itch cytoplasmic-nuclear shuffling.

Efforts are underway to examine the possible contribution of Itch auto-catalytic ubiquitylation to these processes.

A significant number of Itch molecular targets are important regulators of apoptotic cell death [16,8,9]. As a result, their inappropriate removal, due to altered Itch regulation would be likely linked to cellular transformation. It is therefore crucial to unveil the physiological relevance of Itch self-ubiquitylation.

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