The ubiquitin ligase Itch is auto-ubiquitylated in vivo and in vitro but is protected from degradation by interacting with the deubiquitylating enzyme FAM/USP9X*

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Itch is a ubiquitin ligase that has been implicated in the regulation of a number of cellular processes. We previously have identified Itch as a binding partner for the endocytic protein Endophilin and found it to be localized to endosomes. Using affinity purification coupled to mass spectrometry, we have now identified the ubiquitin-protease FAM/USP9X as a binding partner of Itch. The association between Itch and FAM/USP9X was confirmed in vitro by glutathione S-transferase pulldown and in vivo through communoprecipitation. Itch and FAM partially colocalize in COS-7 cells at the trans-Golgi network and in peripheral vesicles. We mapped the FAM-binding domain on Itch to the WW domains, a region known to be involved in substrate recognition. However, transient overexpression of FAM/USP9X resulted in the deubiquitylation of Itch. Moreover, we show that Itch auto-ubiquitylation leads to its degradation in the proteasome. By examining the amounts of Itch and FAM in various cell lines and rat tissues, a positive correlation was found in the expression of both proteins. This observation suggests that the levels of FAM expression could have an influence on Itch in cells. Experimental decrease in FAM levels by RNA interference leads to a significant reduction in intracellular levels of endogenous Itch, which can be prevented by treatment with the proteasome inhibitor lactacystin. Accordingly, overexpression of FAM/USP9X resulted in a marked increase in endogenous Itch levels. These results demonstrate an intriguing interplay between a ubiquitin ligase and a ubiquitin protease, based on direct interaction between the two proteins.

The ubiquitin-mediated proteolytic pathway has been implicated in multiple cellular processes, including endocytosis, cell cycle regulation, transcriptional activation, and antigen presentation. Polyubiquitylated proteins are recognized and degraded by the proteasome, a multisubunit protein degradation complex (1). Protein ubiquitylation also serves regulatory functions in the cell that do not involve proteasome-mediated degradation. For example ligand-induced ubiquitylation of the Ste2 receptor in yeast triggers receptor endocytosis and targeting to vacuoles (2). Similarly, ubiquitylation of the epidermal growth factor receptor (EGFR) triggers its degradation in lysosomes after trafficking in the endosomal compartments (3).

The ubiquitin ligase Itch belongs to the Nedd4/Rsp5p HECT domain-containing family of E3 ligases characterized by a number of regions that are involved in intracellular localization and protein-protein interactions, which include an N-terminal C2 domain, four WW domains, and a C-terminal HECT domain (Fig. 1A). The N-terminal Itch C2 domain is responsible for its intracellular localization to endosomes (4); the WW domains are responsible for the recognition of several substrate proteins (e.g. Refs. 5–12); the C-terminal HECT domain harbors the ligase activity of the enzyme (13) (Fig. 1A).

Previously we have demonstrated that Endophilin A1, an Src homology 3 (SH3) domain-containing protein that functions in clathrin-mediated endocytosis is a substrate for Itch. Interestingly, Itch does not interact with Endophilin via its WW domains; rather through a short 18-amino acid proline-rich domain (PRD) (4). Because of the prominent role of ubiquitylation in endocytosis and our observation that both Itch and Endophilin are highly expressed in the brain, we performed pulldown experiments from rat brain extracts to identify novel substrates of Itch. Interestingly, one of the protein bands isolated was identified as the ubiquitin specific protease FAM, also known as USP9X.

Ubiquitin proteases, or deubiquitylating enzymes, form a large class of proteins whose activity is to mediate the disassembly of ubiquitin-protein complexes. For example, the deubiquitylating enzyme A20 has been shown to be localized to the trans-Golgi network, where it can interact with ubiquitin-mediated substrates (14). This interaction results in the removal of ubiquitin, thus preventing further degradation of the substrate by the proteasome. In contrast, for many substrates it is the ubiquitin ligase that is responsible for the recognition of proteasome substrates, as the deubiquitylating enzyme is required for the removal of ubiquitin after degradation. The interaction between ubiquitin ligases and deubiquitylating enzymes is an important and often intricate regulation of protein degradation (15). It is not yet clear whether the interaction between Itch and FAM results in a similar interaction in vivo. However, it is possible that Itch is a regulator of FAM levels in vivo, thus preventing its degradation by the proteasome.

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§§ The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; PRD, proline-rich domain; USP, ubiquitin-specific protease(s); siRNA, small interfering RNA; GFP, green fluorescent protein; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; MS/MS, mass spectrometry; CL, cell lysate(s); JNK, c-Jun N-terminal kinase; WT, wild type.
bly of ubiquitin-protein conjugates. Based on sequence motif similarity, more than 90 deubiquitylating enzymes have been identified in the human genome and were originally classified into two main families: the ubiquitin C-terminal hydrolases and ubiquitin-specific proteases (USP) (14). Proteases belonging to the C-terminal hydrolase family are generally small (20–30 kDa) and are associated with the proteasomes. They are involved in the generation/recycling of ubiquitin monomers from polyubiquitin chains by removing it from proteins degraded in proteasomes (15). Members of the USP family, which includes FAM/USP9X, are large (60–300 kDa) and are able to hydrolyze ubiquitin from ubiquitylated substrates prior to proteasomal entry, thus extending the half-life of the protein and therefore modulate their activities in the cell. This type demonstrates tissue specific pattern of expression and has been shown to exhibit substrate specificity.

USP9X/FAM is the mammalian counterpart of the product of the *Drosophila* fat-facets gene (*fjf*) that is essential for normal eye development and viability of the early embryo (16). Genetic experiments have identified the Liquid facets protein (Lqf) as one critical substrate of Faf in the eye (17). In mammalian cells, a few substrates have been identified for USP9X/FAM. It binds to and stabilizes β-catenin and AF-6, important components of cell-cell adhesions and members of a transcription factor complex. Another substrate for FAM/USP9X is the vertebrate homologue of Lqf, Epsin1 (17–19). Epsin 1, a major component of cell-cell adhesions and members of a transcription factor complex. Another substrate for FAM/USP9X is the vertebrate homologue of Lqf, Epsin1 (17–19). Epsin 1, a major binding partner for Eps15 (EGFR-phosphorylated protein 15), functions as an important adaptor in clathrin coat assembly and may have additional roles in growth factor receptor signaling and transcription (20).

In this report, we characterize the interaction between Itch and FAM/Usp9X. Similar to other HECT domain ubiquitin ligases, Itch activity leads to its auto-catalytic ubiquitylation (21–23). We show that this auto-ubiquitylation activity leads to proteasomal degradation of Itch. Interaction between Itch and FAM reverses Itch auto-ubiquitylation and protects the ligase from proteasomal degradation. Consistent with this observation, experimental manipulation of FAM levels by overexpression or siRNA-induced down-regulation directly impact on endogenous Itch levels. Accordingly, the expression levels of both proteins are correlated in a variety of tissues and cell lines. Together, these results suggest that FAM directly regulates the level of expression of Itch, which suggests that Itch-dependent cellular processes would also be dependent on FAM expression.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs and Mutagenesis—Full-length Itch WT, Itch ΔC2, Itch ΔC2/ΔPRD, Itch C2, Itch PRD, and ligase-dead Itch CA constructs were described in Angers et al. (4). Other Itch cDNA constructs were obtained by PCR using Vent DNA polymerase (New England Biolabs) and the following primers: Itch HECT, 5'-GAGAATTCGATAAGTCGGACCTATCTGAG-3' (forward) and 5'-GAGAGGGTACCGGCACCTCAAGTCTCGAG-3' (reverse); Itch ΔHECT, 5'-GGAATTCGATAAGTCGGACCTATCTGAG-3' (forward) and 5'-GAGAGGGTACCGGCACCTCAAGTCTCGAG-3' (reverse); Itch WW, 5'-GGAATTCGATAAGTCGGACCTATCTGAG-3' (forward) and 5'-GAGAGGGTACCGGCACCTCAAGTCTCGAG-3' (reverse); Itch WW, 5'-GGAATTCGATAAGTCGGACCTATCTGAG-3' (forward) and 5'-GAGAGGGTACCGGCACCTCAAGTCTCGAG-3' (reverse). The resulting PCR products were subcloned into pGEX-4T-1 (Amersham Biosciences) to add a GST tag. Itch ΔWW was produced by deleting the 5′ end of Itch full-length in pGEX-4T-1 with EcoRI digestion and subcloning in its place the PCR product obtained from primers forward 5'-GGAATTCGATAAGTCGGACCTATCTGAG-3′, reverse 5'-GGAATTCGATAAGTCGGACCTATCTGAG-3′. Itch ΔWW was then centrifuged at 150,000 × g for 30 min. For immunoprecipitation and pulldown assays, extracts of transfected cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (20 mM NaH2PO4, 0.9% NaCl, pH 7.4) for 10 min, permeabilized with 0.2% Triton X-100/phosphate-buffered saline for 4 min and processed with appropriate primary and secondary antibodies.

**Immunoprecipitation and Pulldown Assays**—For cultured cells, 100-mm dishes of transfected HEK-293T cells were washed in phosphate-buffered saline and resuspended in 1 ml/dish buffer A (20 mM HEPES, pH 7.4, 150 mM NaCl, protease inhibitors). The cells were sonicated and Triton X-100 was added to a final concentration of 1%. Extracts were incubated for 20 min at 4 °C and centrifuged at 13,000 rpm in a microcentrifuge. For brain extracts, adult rat brains were homogenized in buffer A and centrifuged at 1,000 × g for 10 min. Triton X-100 was added to the supernatant to a final concentration of 1%, and the extract was incubated for 30 min at 4 °C. The sample was then centrifuged at 150,000 × g for 30 min. For immunoprecipitation assays, extracts of transfected cells were pre-cleared with either protein A-Sepharose or protein G-agarose beads. The pre-cleared supernatants were then incubated with the indicated antibodies and protein A-Sepharose or protein G-agarose beads. For brain extracts, the samples were processed with appropriate primary and secondary antibodies.
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10 μg of the appropriate GST fusion protein for 4–16 h at 4 °C. Beads were washed extensively in the same buffer and prepared for Western blot analysis.

In Vitro Ubiquitination Assays—Recombinant ubiquitin was purchased from Sigma, and ubiquitin-activating enzyme (E1) and UbcH7 (E2) were purchased from Affinity Bioreagents. Full-length Itch CA proteins were produced as GST fusions and eluted from glutathione beads by thrombin cleavage. Ubiquitination reactions (25 μl) contained 10 μg of Itch CA, ubiquitin (25 μm), ubiquitin-activating enzyme E1 (100 nm), ubiquitin-conjugating enzyme UbcH7 E2 (0.5 μm), and 10 μg of total proteins extracted from HEK-293T cells, or the same extract immunodepleted for Itch, and ATP (4 mM) in 50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, and 1 mM dithiothreitol. The reactions were incubated for 90 min at 25 °C and stopped by addition of SDS-PAGE loading buffer and boiling. The reaction mixtures were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot.

RESULTS

Identification of FAM/USP9X as a Novel Itch-interacting Partner—To detect Itch binding partners, GST and GST-Itch fusion proteins precoupled to glutathione-Sepharose were then incubated with rat brain extract. Affinity-purified complexes were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue G (Fig. 1B). Several protein bands that specifically associated with GST-Itch were identified and cut out of the gel. Each band was individually processed to yield in-gel trypsin digestion products and the resulting peptide products were separated and analyzed in an automated system by nanoscale liquid chromatography quadrupole time-of-flight MS/MS as described in Ref. 28. Spectra were analyzed by MASCOT software to identify tryptic peptide sequences matching to the National Center for Biotechnology Information of non-redundant protein data base with a confidence level of 95% or greater (29). Six of the eight bands isolated were heavily contaminated with GST and/or Itch peptides, which prevented the identification of potential Itch interacting partners present within that region of the gel. One of the remaining bands, positioned at ~40 kDa in the gel, contained 12 peptides assigned to Endophilin. Itch was identified as an Endophilin binding partner in earlier work (4). The other band was positioned well above the 200 kDa marker. MS/MS analysis revealed 28 peptide matches to the ubiquitin specific protease USP9X/FAM (Fig. 1B). The calculated molecular mass of USP9X/FAM is ~290 kDa.

FAM/USP9X Directly Forms Complexes with Itch—FAM/USP9X is a large protein and the conserved enzymatically active site represents only a small fraction of its size. It is thus likely that the protease exhibits a complex structure, possibly with multiple specific binding domains to recognize various substrates (30, 31). To verify that FAM/USP9X and Itch form complexes in vivo at physiological concentrations, we performed coimmunoprecipitation experiments of endogenous proteins in cell lysates from HEK-293T cells. HEK-293T cells were selected because they express detectable levels of both Itch and FAM/USP9X (Fig. 6B). Immunoprecipitation of endogenous Itch coimmunoprecipitated substantial amounts of FAM/USP9X (Fig. 2A). Endophilin was also coimmunoprecipitated. Hence endogenous Itch is capable of interacting with both FAM/USP9X and Endophilin. Since it is known that Endophilin directly interacts with Itch, we next investigated whether coimmunoprecipitation of Itch with Endophilin would also pull down FAM/USP9X. Indeed, immunoprecipitation of endogenous Endophilin resulted in the coimmunoprecipitation of both FAM/USP9X and Itch (Fig. 2A). Taken together, these results suggest that Itch can interact with both Endophilin and FAM/USP9X, in the same ternary complex in vivo.

We next performed overlay experiments to determine whether the interaction between Itch and FAM/USP9X is direct. In these assays, extracts from HEK-293T cells were
resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with purified GST, GST-Itch, or GST-Endophilin. Bound proteins were subsequently detected with an anti-GST antibody. Significant binding of Itch to FAM/USP9X was observed. Additionally, Itch bound directly to Endophilin (Fig. 2B). On the other hand, Endophilin did not directly interact with FAM/USP9X under these conditions but with several other proteins migrating around 100 kDa, most likely corresponding to Itch, GLK, and Dynamin, proteins previously demonstrated to interact with Endophilin (Fig. 2B) (4, 32, 33). No binding was detected when resolved HEK-293T proteins were incubated with GST alone. Thus, the interaction between Itch and FAM can occur in the absence of an intermediate. However, results from the coimmunoprecipitation and overlay assays indicate that the association between FAM/USP9X and Endophilin can be explained via an indirect interaction, most likely mediated by Itch. These data also imply that FAM/USP9X exhibits specificity in its direct interaction with select substrates.

To further confirm the interaction between the proteins, we investigated whether FAM/USP9X and Itch are localized to similar subcellular compartments. The subcellular localization of Itch and FAM/USP9X have been previously explored separately. In COS-7 cells Itch expression was found in the trans-Golgi network and endosomal compartments (4). The perinuclear pool overlapped with markers of the Golgi with no significant overlap with those of the endoplasmic reticulum. Similar to Itch localization, FAM/USP9X staining was associated with the Golgi, multivesicular bodies, and lysosomes. Little, if any, colocalization was found with markers of the early endosomes or the endoplasmic reticulum (27). To explore this further, we transiently transfected COS-7 cells with GFP-tagged Itch-full length and performed immunofluorescent detection of endogenous FAM/USP9X using a specific FAM polyclonal antibody (27) (Fig. 2C). Similar to previous reports, FAM staining was visualized predominantly in a perinuclear compartment and exhibited punctate staining that extended throughout the cell. Similarly, the expression of Itch was predominantly detected as punctate structures in the cytoplasm and around the nucleus, consistent with our previous observations. The proteins overlapped in a perinuclear compartment most likely corresponding to the Golgi apparatus. The extent of colocalization is less appar-

![FIGURE 2. Itch forms complexes with FAM/USP9X. A, coimmunoprecipitation (IP) of endogenous Itch (left panel) or Endophilin (right panel) proteins. CL from HEK-293T cells were immunoprecipitated with antibodies against either Itch or Endophilin. CL (100 μg) and immunoprecipitated complexes were separated on a 3–12% SDS-PAGE. The gel was cut horizontally and Western blotted with the FAM/USP9X antibody (top), the Itch antibody (middle), and the Endophilin antibody (bottom). As a control (C), protein A-Sepharose beads were incubated with the CL in the absence of either antibody. IB, immunoblotted. B, overlay assay using GST, GST-Itch, or GST-Endophilin were performed on extracts from HEK-293T cells. 100 μg of CL from HEK-293T cells was separated on SDS-PAGE. The membrane corresponding to each lane was cut vertically, incubated with 50 pmol/ml GST, GST-Itch, or GST-Endophilin separately, and Western blotted with antibodies to GST. The first lane shows control blots with the indicated antibodies. C, GFP-Itch was transfected into COS-7 cells, and its localization was compared with that of endogenous FAM/USP9X. The colocalization of Itch (green) with FAM/USP9X (red) is revealed in the blended image (right panel). Open arrows point to the trans-Golgi network region where most colocalization was observed. The closed arrows point to vesicular compartments where both proteins are also present. n = nucleus. Scale bar represents 10 μm.]

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A

WT(9-862)

ΔC2(104-862)

ΔC2/ΔPRD(230-862)

HECT(482-862)

ΔHECT(9-485)

C2(9-188)

PRD(154-235)

WW(230-465)

ΔPRD/WW(184-485)

ΔWW(9-277,452-962)

B

FAM

Endophilin

CL

GST

WT

ΔC2

ΔC2/ΔPRD

HECT

ΔHECT

C2

PRD

WW

ΔPRD/WW

ΔWW

FIGURE 3. Interaction of FAM and Itch through the WW domain. A, schematic illustration of full-length WT GST-Itch and the deletion mutants constructs: GST-Itch lacking the C2 domain (ΔC2), GST-Itch lacking the C2 and proline rich domain (ΔC2/ΔPRD), GST-Itch HECT domain fusion protein, GST-Itch lacking the ligation domain HECT (ΔHECT), GST-Itch fusion protein containing the C2 alone (C2), GST-Itch fusion protein containing the PRD alone (PRD), GST fused to the WW domain of Itch only (WW), GST-Itch fusion containing the C2 and PRD domain (C2/PRD), GST-Itch fusion containing the PRD and WW domain (PRD/WW), and GST-Itch lacking the WW domains (ΔWW). B, soluble brain extracts (CL) were mixed with the GST alone, GST-Itch WT, or the various GST-Itch mutants, precoupled to glutathione-Sepharose beads. The bound proteins were then subjected to SDS-PAGE followed by Western blot analysis using anti-endogenous FAM and Endophilin.

All GST-Itch fusion proteins containing the WW domains were able to pull down FAM/USP9X from the extract. Conversely those lacking the WW domains were unable to bind FAM/USP9X (Fig. 3B). Deletion of the WW domains alone completely abolished the ability of Itch to bind to FAM/USP9X (Fig. 3B). Similar to previous reports, Itch required the PRD domain to recognize Endophilin (Fig. 3B). Additionally, FAM/USP9X did not bind to control GST (Fig. 3B) or GST-Endophilin (data not shown). These results demonstrate that FAM/USP9X is recognized by the WW domains of Itch.

FAM/USP9X Can Deubiquitylate Itch—FAM/USP9X is a member of the USP family of deubiquitylating enzymes characterized by a number of conserved regions including the Cys and His boxes in the catalytic core of the enzyme. The protease has been shown to deubiquitylate a number of target proteins such as Epsin and AF-6. On the other hand, Itch itself ubiquitylates various substrates. By virtue of the direct interaction between Itch and FAM/USP9X, we reasoned that each might be a substrate for the other, that is that FAM/USP9X could deubiquitylate Itch, and Itch could ubiquitylate FAM/USP9X. Since Itch can undergo autoubiquitylation (see below), HEK-293T cells were cotransfected with plasmids expressing Myc-ubiquitin and FLAG-Itch with or without V5-FAM. Forty-eight hours post-transfection, the cells were lysed, and ubiquitylated proteins were immunoprecipitated with an anti-Myc antibody. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-FLAG or anti-V5 antibody to assess the presence of Itch or FAM, respectively, in the ubiquitylated protein pool. When cells were transfected with FLAG-Itch and Myc-ubiquitin, ubiquitylated forms of Itch could be detected by the FLAG antibody in the immunoprecipitated fraction (Fig. 4A, lower panel). In the presence of FAM/USP9X, all ubiquitylated FLAG-Itch was lost. Interestingly, ubiquitylated FAM could not be detected in the cells coexpressing FLAG-Itch and V5-FAM, suggesting that Itch might not ubiquitylate FAM (Fig. 5A, upper panel). FLAG Western blots of cell lysates (CL) revealed equal expression of FAM-Itch in all conditions. To further explore FAM-induced deubiquitylation of Itch, HEK-293T cells transfected with FLAG-Itch and Myc-ubiquitin and with or without V5-FAM were immunoprecipitated with anti-FLAG antibody, and immunoprecipitated proteins were blotted with monoclonal anti-Myc antibody to detect ubiquitylation. A significant decrease in Itch ubiquitylation was observed upon FAM/USP9X overexpression represented by a reduction in the number of Itch migrating species resulting from ubiquitin modifications of this protein (Fig. 4B).

Together these results demonstrate that FAM/USP9X is capable of cleaving ubiquitin chains from the ligase Itch.

Itch Is Auto-ubiquitylated and Degraded in the Proteasome—Having established an interaction between Itch and FAM/USP9X, we next sought to address the relevance of this interaction at the cellular level. Previously, the association between ubiquitin ligases and ubiquitin proteases has been shown to stabilize the ligase, which sometimes undergo auto-ubiquitylation followed by proteasome degradation (35, 36). This is consistent with our observation that FAM/USP9X readily deubiquitylates Itch. In light of such an observation we assessed the ability of Itch to ubiquitylate itself.

FAM/USP9X Interacts with Itch through the WW Domain—HECT domain ubiquitin ligases of the Nedd4 family generally interact with their substrates through the WW domains, with recognition preference for the motif PPXY(PY) (34). Additionally, in earlier work we have shown that another domain on Itch, the PRD, is also involved in protein interaction, specifically to Endophilin (4). Hence, we sought to map the specific region on Itch involved in binding to FAM/USP9X. A number of N- and C-terminal truncations were engineered by successively deleting each characterized domain separately or in combination (Fig. 3A). Rat brain extracts were mixed with the constructs expressed as GST fusion proteins precoupled to glutathione-Sepharose. Bound proteins were then resolved on SDS-PAGE and blotted with a polyclonal antibody specific to FAM/USP9X. As a control for Itch interaction, we also blotted for Endophilin.

In more peripheral vesicular structures, suggesting that only a subset pool of both proteins are colocalized in vivo.

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B, soluble brain extracts (CL) were mixed with the GST alone, GST-Itch WT, or the various GST-Itch mutants, precoupled to glutathione-Sepharose beads. The bound proteins were then subjected to SDS-PAGE followed by Western blot analysis using anti-endogenous FAM and Endophilin.
Toward this end, we coexpressed FLAG-Itch and Myc-ubiquitin in HEK-293T cells and subjected Myc immunoprecipitates to Western blot analysis with FLAG antibody and, conversely, blotted FLAG immunoprecipitates with anti-Myc antibodies. Ubiquitylated Itch can be easily observed as the accumulation of higher molecular weights bands (Figs. 4B and 5A). Surprisingly, a mutant Itch construct lacking the ubiquitin ligase activity (ItchC830A) (4) is also ubiquitylated under similar conditions (Fig. 5A). Ubiquitylation of the ligase-inactive mutant is much lower than the WT form, but this is expected given that in these conditions the only source of ligase activity is the endogenous enzymes. This raises the possibility that Itch may be the substrate of other ubiquitin ligases or that it is ubiquitylated in trans by endogenous Itch present in HEK-293T cells. To address this question, we performed in vitro ubiquitylation assays using recombinant Itch-CA produced as a GST fusion protein in E. coli and subsequently cleaved from GST with thrombin. Recombinant Itch CA was incubated with purified mammalian ubiquitin-activating enzyme (E1), recombinant UbcH7 (E2), ubiquitin, and ATP, in the presence of HEK-293T cell extract as a source of E3 ligase. As expected, the ligase inactive form of Itch was ubiquitylated under these conditions. To verify if the ligase activity was provided by Itch itself, we immunodepleted the HEK-293T cell extract with a monoclonal Itch antibody and used the depleted extract as a source of E3 ligase activity. When Itch was eliminated form the cell extract in this manner, no ubiquitylation of Itch CA

FIGURE 4. FAM/USP9X can deubiquitylate Itch. A and B, FLAG-Itch and Myc-ubiquitin were coexpressed in HEK-293T cells either with or without V5-FAM indicated by (+ or −). Lysates were immunoprecipitated (IP) with anti-Myc antibody and immunoblotted (IB) with antibodies to V5 (top) and FLAG (bottom). The expression of FLAG-Itch and V5-FAM in the cell lysates is also shown and represented by CL. B, lysates of similarly transfected cells were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted (IB) with antibodies to Myc.

FIGURE 5. Itch is ubiquitylated by itself and degraded in the proteasome. A, HEK-293T cells were transfected with Myc-ubiquitin and FLAG-Itch WT or the ligase dead mutant FLAG-Itch CA. 48 h post-transfection, cell lysates were obtained and immunoprecipitated (IP) with either anti-FLAG or anti-Myc antibodies as indicated and immunoblotted with either anti-FLAG or anti-Myc antibodies as indicated. Ubiquitylated Itch WT was readily detectable, whereas only a trace of ubiquitylated Itch CA could be observed. B, to determine whether Itch could be ubiquitylated by itself in a trans-molecular fashion, we incubated recombinant Itch CA obtained from a GST fusion protein expressed in E. coli with ubiquitin, E1, E2, and ATP in the presence of total cell lysate or lysate immunodepleted for Itch at 25 °C for 90 min. The reaction mixture was prepared for immunoblot (IB) with anti-Itch antibody. Ubiquitylated Itch (Ub-Itch CA) is indicated. The bottom panel shows the presence and absence of Itch in control and depleted cell lysates, respectively. C, HEK-293T cells transfected with Myc-ubiquitin and GFP-Itch WT were incubated with lactacystin for 16 h or in control media. Lysates were then immunoprecipitated with anti-GFP antibody and immunoblotted with anti-Myc antibody (upper panel) to show ubiquitylation of anti-GFP (lower panel) to show total immunoprecipitated GFP-Itch protein. D, untransfected cells were incubated for 16 h with lactacystin or left in control solution. Total cell lysates were prepared for immunoblotting with anti-Itch antibody and anti-actin antibody to show the amount of loaded proteins.
could be obtained in vitro. (Fig. 5B). The lower panel shows the efficacy of the depletion procedure by blotting the cell extracts with an anti-Itch antibody.

Ubiquitination frequently serves as a signal for proteasome-dependent degradation. To determine whether ubiquitylated Itch undergoes proteasomal degradation, we treated HEK-293T cells transfected with FLAG-Itch and Myc-ubiquitin with the proteasome inhibitor lactacystin. The amount of ubiquitylated Itch immunoprecipitated from treated cells was significantly higher compared with untreated, demonstrating that ubiquitylated Itch is degraded in the proteasome (Fig. 5C). To verify that endogenous Itch was also degraded in proteasomes, HEK-293T cells were transfected with Myc-ubiquitin and incubated in the presence of lactacystin. Cell lysates were then subjected to Western blot analysis using endogenous mouse Itch monoclonal antibody. Levels of endogenous Itch increased in cells treated with lactacystin. Hence, similar to results from our overexpression experiments, endogenous Itch is degraded in a proteasome-dependent manner. (Fig. 5D).

Comparison of Itch and FAM/USP9X Expression in Various Rat Tissue Extracts and Cell Lines—If Itch undergoes proteasomal degradation as a consequence of its own enzymatic activity and that FAM can reverse this effect by deubiquitylating Itch, we reasoned that the presence of FAM might be required to ensure high levels of Itch expression. We thus examined the amounts of both the protease and ligase by carrying out Western blot analysis from rat tissue extracts, which included the brain and a number of peripheral organs. Expression of FAM/USP9X was highest in the whole brain, thymus, and in the lung. Detectable levels of the protease were present in the heart, ovaries, pancreas, and spleen. It was absent, however, in the liver and kidney (Fig. 6A). Itch on the other hand appears to be ubiquitously expressed in both the central and peripheral tissues. However, as expected, its levels were elevated in those organs expressing FAM/USP9X. Hence, in accordance to previous reports, FAM/USP9X appears to have a restricted pattern of expression compared with the ligase Itch; however, levels of the ligase are tightly associated with those of the protease. Similarly, endogenous Itch protein levels paralleled the amounts of FAM/USP9X in various cell lines, which constitute a more homogenous sample than tissue extracts (Fig. 6B).

Interference with FAM Function Significantly Affects Intracellular Levels of Endogenous Itch—To strengthen the conclusion that FAM/USP9X stabilizes Itch in cells, we explored the effects of down-regulating FAM/USP9X expression through siRNA
experiments in HEK-293T cells. A 4-day incubation with various concentrations of two different vectors expressing siRNAs specific for FAM (25) strongly reduced the levels of endogenous FAM/USP9X expression particularly at 15 and 20 μg of plasmid (Fig. 7A, upper panel). As a control, transfection with the plasmid vector expressing a random filling sequence did not result in any changes of FAM/USP9X expression. Accordingly, we detected a diminution in endogenous Itch levels in cells transfected with FAM/USP9X targeted siRNA but no change in β-actin (Fig. 7A, middle and lower panels). The most likely explanation for our findings is that the decrease in Itch expression is due to an increase in the ubiquitylated state of the protein which would serve as a signal for proteasomal degradation. Additionally, these findings strongly suggest that Itch is deubiqui-
ylated by FAM/USP9X in vivo. Since ubiquitylation of pro-
teins is usually associated with their turnover (37), we next examined the effect of proteasomal inhibition on Itch stability. HEK-293T cells were transfected with the two different FAM/USP9X-specific siRNA plasmids separately, along with the control vector. On the third day post-transfection cells were incubated with or without lactacystin for 24 h. Proteasomal inhibition almost completely restored the levels of Itch in the cell and blocked Itch-dependent proteasomal degradation (Fig. 7B). This is comparable with previous reports showing that proteasome inhibition leads to the stabilization of proteasome-targeted ubiquitylated proteins (6, 38). In support of this idea, we reasoned that overexpression of FAM/USP9X should result in further stabilization of endogenous Itch. HEK-293T cells were transfected with vectors expressing Myc-ubiquitin with or without FAM/USP9X. Cell extracts were then blotted with anti-FAM/USP9X and anti-Itch antibodies. The introduction of exogenous protease resulted in increased levels of Itch. These increases appeared to be correlated with the level of exogenous FAM/USP9X expressed (Fig. 7C). These results indicate that the fate of Itch is tightly controlled by the state of FAM/USP9X in the cell. Hence FAM/USP9X plays a critical role in modifying Itch function through deubiquitylation.

It has been previously reported that the stress-related kinase c-Jun N-terminal kinase (JNK) can phosphorylate Itch and that JNK-induced phosphorylation is concomitant to an increase in Itch auto-ubiquitylation in T cells (21). It was also shown by the same group that JNK phosphorylates residues located in the PRD domain of Itch and impacts on the ability of Itch to bind to its target proteins (39). We therefore examined the impact of JNK activity in the capacity of Itch to bind to FAM/USP9X. We overexpressed FLAG-JNK in HEK-293T cells. Overexpression of JNK leads to its autoactivation, as shown by the phospho-JNK blot of cell lysates (Fig. 7D). When JNK is active, immuno-
precipitation of endogenous Itch fails to communoprecipitate FAM/USP9X (Fig. 7D). This is consistent with the observed augmentation of Itch ubiquitylation in response to JNK activation reported by (21).

**DISCUSSION**

The ubiquitin ligase Itch plays key roles in different cellular contexts by virtue of its target substrates. In the present study, we establish that Itch is a substrate for the protease FAM/USP9X. Both proteins appear to specifically and directly inter-

**B. A. Azakir and A. Angers, unpublished observations.**
sory proteins such as Eps15 (45, 46). Epsin is required for endocytosis in yeast and in mammalian cells (20). In mammalian cells Epsin1 has been demonstrated to be a physiological substrate for FAM/USP9X (19). Interestingly, in vitro experiments demonstrated that ubiquitylation of Epsin decreased its binding toward its endocytic-interacting partners such as liposomes, clathrin, and AP-2 thus highlighting a critical role for FAM/USP9X (19). Itch has been directly linked with the endocytic machinery as well, particularly through its interaction with Endophilin and cbl (4, 6).

The critical assembly of the Cbl/CIN85-Endophilin molecular complex at the tyrosine residues of the C terminus of the EGF receptor following ligand activation mediates its ubiquitylation followed by internalization (47). The complex includes Cbl proteins that are RING finger domain-containing E3 ubiquitin ligases implicated in the ubiquitylation and down-regulation of the EGF receptor at the cell surface (47, 48). Generally, escape from Cbl-mediated ubiquitylation and down-regulation is one common characteristic of receptor tyrosine kinases that have undergone oncogenic deregulation. Mutants forms of Cbl whose RING fingers are defective, and thus unable to ubiquitylate the EGF receptor, have been shown to have transforming activity presumably as a consequence of decreased EGF receptor turnover and increased signaling activity. Accordingly, overexpression of Cbl leads to enhanced ubiquitylation and degradation of the EGF receptor (6, 47, 48). The ubiquitin ligase Itch has been shown to bind to and ubiquitylate Cbl resulting in its degradation in proteasomes. Consequently, Itch inhibits Cbl-mediated ubiquitylation and down-regulation of the EGF receptor resulting in an increase in EGF receptor levels (6). Another component of this complex is the adaptor protein Endophilin that participates at multiple stages in clathrin-coated endocytosis. As mentioned earlier, we have shown that Itch can also bind to and ubiquitylate Endophilin (4). Moreover there is evidence to suggest that Eps15, another protein implicated in EGFR internalization, is also a substrate for Itch (49).

In conclusion we have identified a novel key mechanism by which Itch levels are normally regulated in the cell. Our findings demonstrate that Itch is auto-ubiquitylated, and protected from degradation by the ubiquitin protease FAM/USP9X. It now becomes of great interest to determine which pathological and physiological states are influenced by the physical and functional interaction between both proteins.

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