Cancer Research

Negative Regulation of the Hippo Pathway by E3 Ubiquitin Ligase ITCH Is Sufficient to Promote Tumorigenicity

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Abstract

The Hippo tumor suppressor pathway, originally defined in fruit flies, regulates cellular proliferation and survival and exerts profound effects on normal mammalian cell fate and tumorigenesis. The present understanding of Hippo pathway components and mechanisms remains incomplete in cancer. WW domain-containing proteins regulate diverse biological processes through interaction with proline-tyrosine (PPxY)-containing targets. In this study, we report that the E3 ubiquitin ligase ITCH regulates stability of LATS1, a serine/threonine kinase in the Hippo pathway, through protein–protein interaction of the PPxY motifs of LATS1 with the WW domains of ITCH. Ubiquitination of LATS1 catalyzed by ITCH stimulated the proteasomal degradation of LATS1. Furthermore, ITCH-mediated degradation of LATS1 was associated with enhanced cell growth, induction of epithelial-mesenchymal transition, and increased tumorigenicity. Conversely, ITCH depletion increased LATS1 levels, enhancing FAS-induced apoptosis and reducing proliferation, survival, and migration. These phenotypes were rescued when both ITCH and LATS1 were depleted. Together, our results reveal a novel functional link between ITCH and the Hippo pathway, deepening their critical roles in tumorigenesis. *Cancer Res; 71(5); 2010–20. ©2011 AACR.*

Introduction

The size of developing organs is controlled by cell growth, proliferation, and apoptosis, which collectively define organ size and cell number. The Hippo pathway was recently described to control these cellular processes (1, 2). Impairment of these processes leads to human diseases such as cancer (2). Many components of the Hippo pathway were discovered initially in *Drosophila* mosaic genetic screening owing to strong overgrowth phenotypes (3). These components are highly conserved in mammals (4, 5). The current model suggests that the mammalian MST1/2 kinase, complex with a scaffold protein, WW45, phosphorylates and activates LATS1/2 kinase. LATS1/2 kinase directly phosphorylates and inactivates the WW domain-containing transcription coactivators Yes-associated protein (YAP; 6, 7) and its paralog TAZ (8), though other kinases may also be involved (9). Cell–cell contact of cultured mammalian cells induces phosphorylation and inactivation of YAP1, whereas overexpression of YAP1 bypasses contact inhibition growth (6). Importantly, inactivation of YAP and TAZ has been shown to regulate organ size and tumorogenesis (1, 2, 10).

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doi: 10.1158/0008-5472.CAN-10-3516

LATS1/2 is a serine/threonine (Scr/Thr) kinase implicated in cell-cycle regulation. LATS1 and LATS2 inhibit tumor growth by inducing G_2-M and G_1-S arrest, respectively (11–13). In addition, LATS1/2 promotes apoptosis by inducing proapoptotic or downregulating antiapoptotic proteins (11, 12, 14). Mutations or reduced expression, by promoter hypermethylation, of *LATS1* has been observed in a variety of human cancers including sarcomas (15), leukemia (16), astrocytoma (17), and breast (18). Targeted ablation of *Lats1* in mice led to formation of soft-tissue sarcomas and ovarian stromal-cell tumors both spontaneously or in response to carcinogenic treatments (19) furthering its tumor suppressor activity. LATS1 contains a ubiquitin-associated (UBA) domain and 2 PPxY motifs that are known to interact with WW domains (Fig. 1A; ref. 20). WW domains are protein modules that mediate protein–protein interactions through recognition of proline-rich peptide motifs and phosphorylated Ser/Thr-proline sites (21, 22). Recently, MST2 and LATS1/2 were shown to regulate apoptotic function of YAP through PPxY-WW domain interaction (7, 23). These results prompted us to search for other WW domain-containing proteins that might regulate LATS1/2 function.

Protein ubiquitination is a fundamental mechanism for regulating the half-lives and activity of many proteins by targeting them for proteasomal degradation (24). The specificity of ubiquitination reaction is achieved by the E3 ubiquitin ligase (E3). The NEDD4-like family of E3 ubiquitin ligases, such as NEDD4 and ITCH, is characterized by distinct modular domain architecture, with each member consisting of a Ca^{2+}/lipid-binding (C2) domain involved in membrane targeting, 2–4 WW domains conferring substrate specificity, and a

HECT-type ligase domain providing the catalytic E3 activity (24, 25). ITCH was originally identified as a gene disrupted in the non-agouti–lethal 18H or *Itchy* mice that suffer from severe immune and inflammatory defects (26, 27). ITCH contains 4 WW domains known to associate with PPxY containing targets (Fig. 1A). A number of ITCH substrates that have been implicated in tumorogenesis and chemosensitivity have been identified, including c-Jun (28, 29), p73 (30), p63 (31), and ErbB4 (32). Here, we show that the E3 ubiquitin ligase ITCH associates with LATS1 and negatively regulates the Hippo pathway.

Materials and Methods

Cell culture and transient transfection

HEK293, HeLa, and MEF cells were grown in DMEM, supplemented with 10% FBS (Gibco), glutamine, and penicillin/streptomycin (Beit-Haemek). MCF10A cells were grown in DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, and antibiotics. Cells were routinely authenticated, and cell aliquots from early passages were used. Transient transfections were achieved using Mirus TransLTi (Mirus Bio LLC).

Plasmid construction

Full-length cDNAs of YAP2 and LATS1 were cloned into a pCDNA4-HisMaxB vector (Invitrogen) using standard protocols. ITCH constructs were reported previously (31). Flag-LATS2 was a gift from Dr. Tadashi Yamamoto (University of Tokyo, Japan). DsRed-YAP2, EGFP-LATS1, and their mutant variants were described in (23) and obtained through Addgene.

Immunoprecipitation and immunoblot analysis

Four hours prior to cell lysis, cells were treated with 20 μmol/L of MG132 (Sigma Aldrich). Cells were lysed by using Nonidet P-40 lysis buffer containing 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors (33). Lysates were precleared with mouse IgG, immunoprecipitations were carried out in the same buffer, and lysates were washed 4 times with the same buffer containing 0.1% Nonidet P-40. Western blotting was conducted under standard conditions. Antibodies used were monoclonal anti-HA (Covance), monoclonal anti-Omni, polyclonal anti-YAP (Santa Cruz Biotechnology), monoclonal anti-Flag, anti-Flag-HRP (Sigma), polyclonal anti-LATS1/2 (Bethyl), anti-HA-HRP (Roche Applied Science), phospho-YAPS127 (Cell Signaling), monoclonal anti-ITCH (BD Biosciences), and monoclonal anti-Ubiquitin, Lys48-Specific (Millipore).

In vivo ubiquitination assay

HEK293 cells were cotransfected with HA-UB, Max-LATS1 with or without Flag-ITCH or Flag-ITCHC830A. After 24 hours, cells were treated with MG-132 (20 μmol/L) for 4 hours. Lysates were immunoprecipitated using anti-Max antibody, washed 4 times, and immunoblotted with anti-HA-HRP.

Measurement of steady-state and half-life of LATS1 protein level

HEK293T cells were transfected with Max-LATS1 with or without Flag-ITCH. Twenty-four hours posttransfection, cells were lysed or treated with the protein synthesis inhibitor cycloheximide (100 μg/mL) for 3 and 6 hours. Cell lysates were subjected to immunoblotting.

Immunofluorescence

Cells were seeded on round slide-cover slips in 12-well plates. Twenty-four hours later, cells were transfected with the expression plasmids. Twenty-four hours posttransfection, cells were fixed in 3.7% PBS-buffered formaldehyde, permeabilized with 0.05% Triton X-100 at room temperature. Cells were then incubated for 1 hour in 10% goat serum (Invitrogen), with primary antibody for 1 hour and with secondary antibody. Antimouse Texas red-conjugated antibody-647 (Molecular Probes) was used to detect ITCH. Cells were examined by confocal microscopy (Olympus) under 60× magnification.

Three-dimensional culture of MCF10A cells

The test was done as described previously (34). In brief, 3,000 cells were seeded on a solidified layer of growth factor reduced matrigel measuring approximately 1–2 mm in thickness. The cells are grown in an assay medium containing 5 ng/mL EGF and 2% Matrigel. To test for cell survival, the growth medium was depleted of growth factors.

In vivo tumorigenesis

About 5×10^6 cells were injected s.c. on both dorsal sides of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (6/group) and tumor formation and size were followed up for 6–8 weeks, after which animals were sacrificed and tumors excised and weighed.

Statistical analysis

Results were expressed as mean ± SD. The Student *t*-test was used to compare values of test and control samples. $P < 0.05$ indicated significant difference.

Detailed Materials and Methods are included in the Supplementary Information.

Results

ITCH physically interacts with LATS1

Because LATS1 requires the intact WW domains of YAP and TAZ to regulate the Hippo pathway (7, 23), we assumed that other WW domain-containing proteins might regulate LATS1 function, thereby controlling the Hippo pathway. Screening of several WW domain-containing proteins to precipitate LATS1, using commercial WW domain arrays, identified the E3-ubiquitin ligase ITCH as a putative interacting partner (Fig. 1B). As controls, we detected both YAP and TAZ as previously known partners of LATS1 whereas the WW domain-containing proteins DRP2, DMD, and UTRN showed no interaction (Fig. 1B). To confirm our preliminary observation, we cotransfected HEK293 cells with expression vectors encoding Max-LATS1 and Flag-ITCH. Cell lysates

were immunoprecipitated with appropriate antibodies followed by immunoblotting with HRP-conjugated antibodies. Complexes between LATS1 and ITCH were detected in both directions (Fig. 1C). As a control, there were no detectable complexes in anti-IgG immunoprecipitates (Fig. 1C). As LATS2 shares many features with LATS1 and contains one PPxY motif, we examined whether ITCH can precipitate LATS2. Our results revealed no specific complexes between LATS2 and ITCH (Fig. S1A); therefore we proceeded to further investigate ITCH-LATS1 interaction.

To verify physiologic association of ITCH-LATS1, we examined interaction of endogenous proteins following activation of the Hippo pathway. One approach to activate the Hippo pathway is by expressing MST2 kinase that in turn activates LATS1. Therefore, we carried out immunoprecipitation in HEK293 cells in the presence or absence of MST2. We detected stronger endogenous ITCH-LATS1 interaction when MST2 was present (Fig. 1D). Previous *in vitro* studies have shown that cellular contact can also induce the Hippo pathway (6). Therefore, we examined complex formation in high cell culture density. We found that LATS1 co-immunoprecipitated ITCH in high-density MCF7 cell culture (Fig. 1E). We noted the appearance of an additional upper band in self-IP of LATS1 (Fig. 1D and E), the identity of which is to be determined. Nevertheless, in both experiments we observed specific coimmunoprecipitation of endogenous ITCH by LATS1. These results suggest that activation of the Hippo pathway enhances physical association of LATS1 with ITCH.

We next determined whether mutations in the PPxY motifs of LATS1 would result in loss of this interaction. Because LATS1 contains 2 PPxY motifs, we replaced the tyrosine (Y) residues with alanine (A) generating Max-LATSPY1, Max-LATSPY2 and double mutant Max-LATSPY1,2. To determine whether ITCH coimmunoprecipitates LATS1 mutants, we cotransfected HEK293 cells with Flag-ITCH and each of the LATS1 mutants and conducted immunoprecipitation. We found that ITCH coimmunoprecipitates with both single LATS1 mutants at a reduced level compared with wild-type LATS1 (Fig. S1B). Mutation of both PPxY motifs in LATS1, however, inhibited interaction with ITCH (Fig. 1F), suggesting that both PPxY motifs interact with ITCH.

We further mapped WW domain of ITCH that binds LATS1. GST pull-down experiments with single or all 4 ITCH WW domains fused to GST showed that LATS1 predominantly interacted with ITCH WW1 domain (Fig. 1G). Interaction of LATS1 with WW2, WW3, and WW4 was less efficient. These results imply that ITCH, predominantly via its first WW domain, interacts with both PPxY motifs of LATS1.

ITCH ubiquitinates LATS1

To analyze the functional significance of the interaction between LATS1 and ITCH, the role of ITCH in ubiquitination

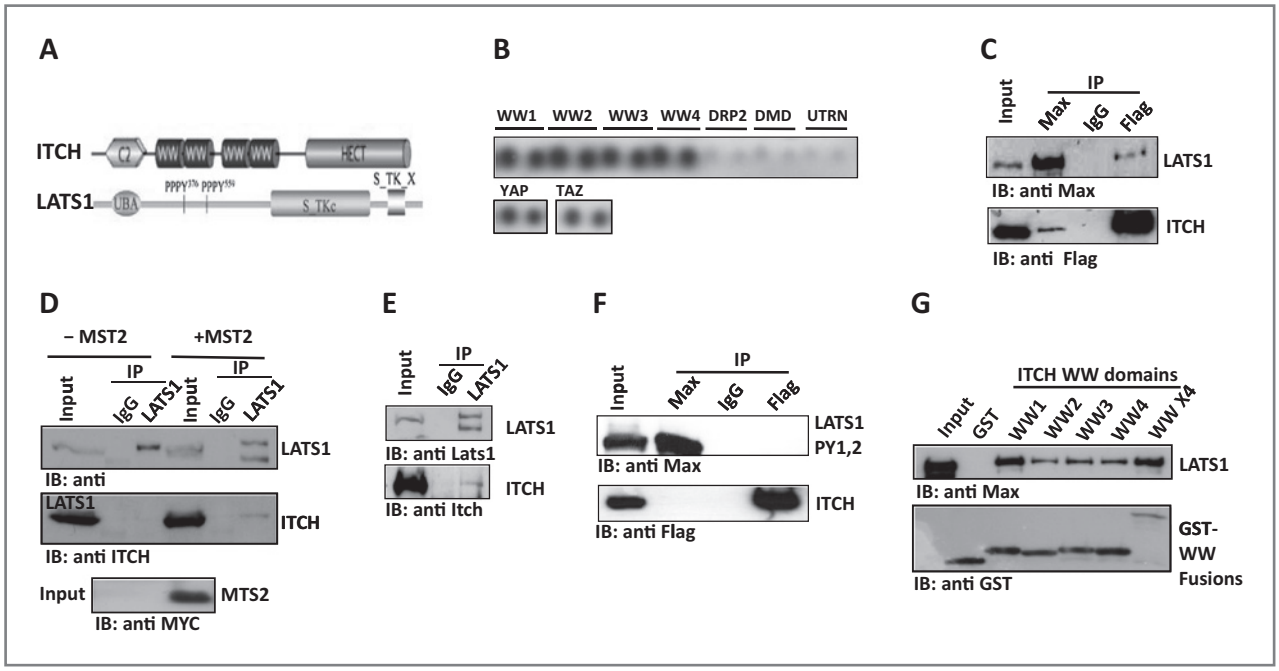

Figure 1. Physical interaction between LATS1 and ITCH. A, schematic representation of the modular structure of the LATS1 and ITCH proteins. B, binding pattern of different WW domain containing proteins to LATS1 using commercial WW domain arrays (Panomics). C, ITCH forms a complex with LATS1 *in vivo*. HEK293 cells were cotransfected with Max-LATS1 and Flag-ITCH. Cell lysates were immunoprecipitated (IP) with indicated antibodies. The immunoprecipitates were analyzed by immunoblotting (IB) as indicated. D and E, endogenous LATS1-ITCH interaction. HEK293 cells transfected with Myc-MST2 or empty vector and MCF7 cells cultured at high-cell density lysates were IP with the indicated antibodies and analyzed by IB. Cells were pretreated with MG132. F, intact PPxY motifs in LATS1 are required for ITCH binding. HEK293 cells were cotransfected with Flag-ITCH and LATS1PY1,2 mutant and treated as shown in C. G, WW domains of ITCH interact with LATS1. GST fusion proteins including one of the WW domains of ITCH, all 4 together or glutathione S-transferase (GST) alone, were incubated with lysates of HEK293 cells expressing Max-LATS1. Complexes were captured with glutathione-Sepharose. Bound protein was detected by anti-Max and then reblotted with anti-GST antibodies.

of LATS1 was analyzed. Extracts of HEK293 cells transfected with plasmids expressing HA-UB, Max-LATS1, and Flag-ITCH, or the catalytically inactive Flag-ITCHC830A mutant were subjected to immunoprecipitation followed by immunoblotting. Expression of ITCH increased ubiquitination of LATS1 unlike the inactive C830A mutant of ITCH (Fig. 2A), which was still capable of binding LATS1 (Supplementary Fig. 1C). Interestingly, LATS1PY1,2 was also ubiquitinated when wild type or mutant ITCH was expressed (Fig. 2A, Supplementary Fig. S1D). Because it has been shown that Lysine-48 (K48)–linked polyubiquitin predominantly, but not exclusively, targets proteins for proteasomal degradation (35), we set out to determine whether LATS1PY1,2 is K-48-polyubiquitinated using a specific antibody that recognize K-48 (35). We found that, whereas ITCH mediated K-48 ubiquitination of LATS1, this specific antibody failed to recognize ubiquitination of mutant LATS1 (Fig. 2B), suggesting that other E3 ligases might ubiquitinate LATS1PY1,2 perhaps without affecting its degradation.

ITCH regulates the stability and half-life of LATS1 affecting YAP phosphorylation

We next decided to investigate whether ITCH mediates ubiquitination-dependent degradation of LATS1. Because most cellular protein degradation is mediated by the proteasomal pathway, we treated HEK293 cells expressing Max-LATS1 and Flag-ITCH with increasing amounts of the proteasome inhibitor MG-132 and determined the protein level of LATS1 by Western blot. As shown in Fig. 2C, treatment with MG132 led to increased protein levels of Max-LATS1 in a dose-dependent manner whereas treatment with the lysosomal inhibitor bafilomycin had no effect on LATS1 levels (Fig. 2D). These data suggest that LATS1 stability is regulated by the proteasomal pathway.

To determine whether ITCH-mediated ubiquitination of LATS1 promotes LATS1 degradation, we measured steady state levels of LATS1 in the presence and absence of ITCH. Expression of wild-type but not mutant ITCH decreased the protein level of wild-type LATS1 (Fig. 2E) but not

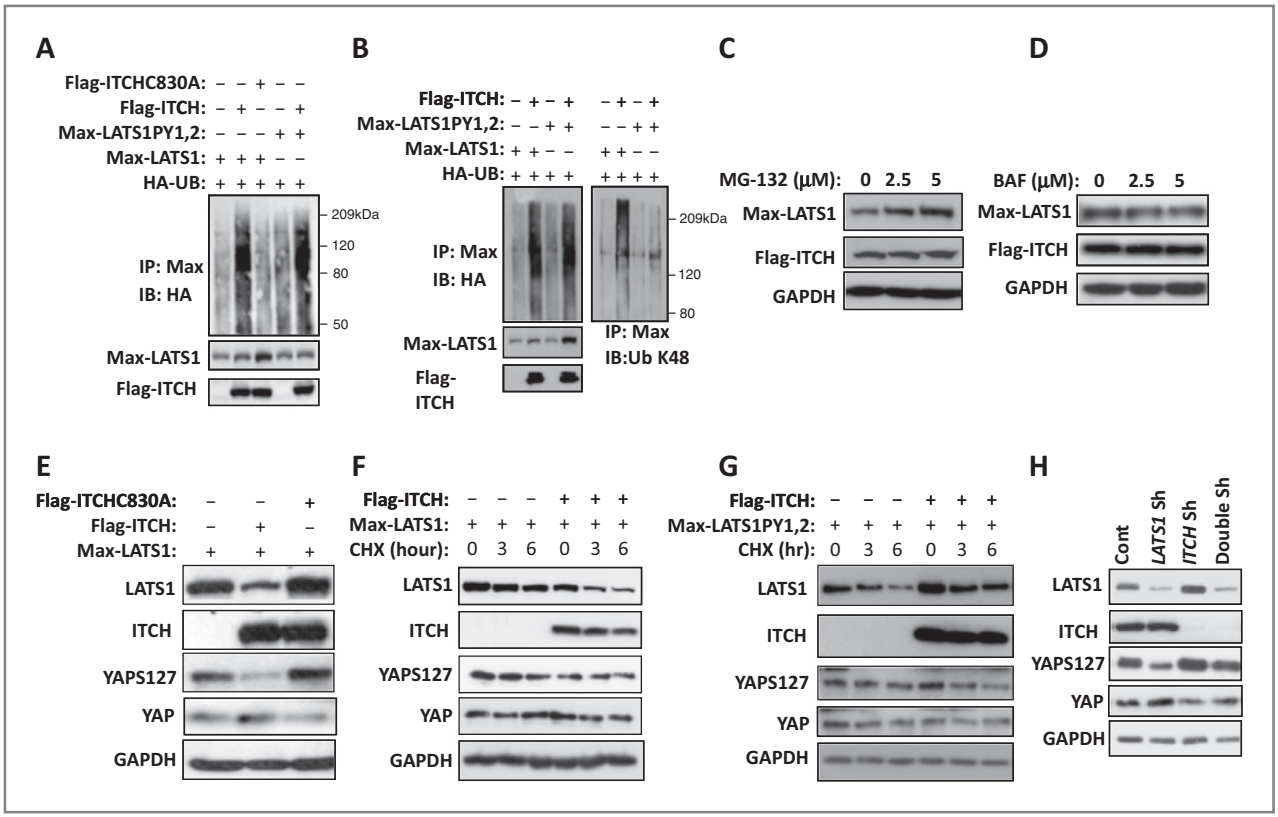

Figure 2. ITCH induces ubiqitination and affects the steady state and half-life of LATS1 protein. A, ITCH ubiquitinates LATS1 *in vivo*. HEK293 cells were transfected with the indicated plasmids. After 24 hours, cells were treated with 20 μmol/L MG132 for 4 hours. Lysate was prepared and IP with anti-Max and detected with anti-HA antibodies (UB). The middle and bottom blots show LATS1 and ITCH levels in the input lysates. B, ITCH ubiquitinates LATS1 but not LATS1PY1,2 using K-48-specific antibody. HEK293 cells were transiently transfected with the indicated plasmids (top) and treated as in A (left). Membranes were reblotted with Ub-K-48-specific antibody (right). C and D, LATS1 stability is controlled by the proteasomal pathway. HEK293 cells were transiently transfected with Max-LATS1 and Flag-ITCH. After 24 hours cells were treated with MG-132 (C), or bafilomycin [BAF (D)] as indicated for 4 additional hours. Equal amounts of total lysates were blotted as indicated. E, ITCH affects the steady state of LATS1 protein. HEK293 cells were cotransfected with the indicated plasmids; 24 hours later, lysates were prepared and analyzed by IB as indicated. F and G, ITCH reduces the half-life of LATS1. HEK293 cells were cotransfected with the indicated plasmids. After 24 hours, cells were treated with 20 μg/mL cycloheximide (CHX) at the indicated time points and analyzed as shown in E. H, effect of ITCH and LATS1 depletion using ShRNA constructs. HeLa cells were transduced with lentiviral-vector of *ITCH* or *LATS1* ShRNA constructs. Lysates were analyzed using the indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

LATS1PY1,2 (Supplementary Fig. S1E). In fact, we observed an increase of mutant LATS1 level suggesting that ubiquitination observed in Fig. 2B might contribute to its stabilization. Because LATS1 interacts with and phosphorylates YAP (7, 23, 36), we next determined whether ITCH-dependent degradation of LATS1 affects YAP phosphorylation using antibody that recognize phosphoprylated serine 127 (S127). Indeed, our results show that LATS1 decreased levels owing to ITCH overexpression are accompanied by decreased phospho-YAP levels and slight increase in total YAP levels (Fig. 2E).

To prove that the ITCH-dependent reduction in LATS1 protein was owing to increased degradation, we analyzed the half-life of LATS1 in the presence or absence of ITCH using the protein synthesis inhibitor, cycloheximide. Whereas ITCH led to shortened half-life of LATS1 and reduced phospho-YAP (Fig. 2F), it increased that of mutant LATS1 (Fig. 2G). To further confirm the importance of ITCH in controlling the degradation of LATS1, we generated stable HeLa cells expressing shRNA constructs specifically targeting the human *ITCH* mRNA and analyzed consequences on LATS1 levels (Fig 2H). We observed that ITCH-depleted HeLa cells displayed higher LATS1 protein levels and phospho-YAP compared with control *shRNA* or *LATS1Sh*-expressing cells (Supplementary Figs. 2A and 2H). Of note, increased phospho-YAP levels were associated with decreased YAP levels. Altogether, these results indicate that ITCH promotes the ubiquitin-dependent proteasomal degradation of LATS1.

Activation of the Hippo pathway induces ITCH and subsequent degradation of LATS1

To further confirm the physiologic role of ITCH-mediated degradation of LATS1, we examined levels of endogenous ITCH following activation of the Hippo pathway. We first examined levels of ITCH in low and high cell density in murine embryonic fibroblasts (MEF). We found that increasing cell density induced ITCH levels (Fig. 3A) though this was not accompanied by downregulation of LATS1. Consistent with published results, YAPS127 phosphorylation was induced (9). To further validate the significance of LATS1 as a target of ITCH, we examined LATS1 levels in *Itch*-knockout (*Itch-/-*) MEF. As expected, LATS1 levels were higher in *Itch-/-* MEF even at lower cell density and this was associated with increased YAPS127 phosphorylation. Culturing *Itch-/-* MEF at high cell density further induced LATS1 levels (Fig. 3A). Similar results were observed when transfecting HEK293 cells with plasmids encoding *FLAG-ITCH* and *MAX-LATS1*. ITCH levels were induced under high cell density conditions and were accompanied by downregulation of LATS1 and YAPS127 phosphorylation (Fig. 3B).

Next, we examined whether ITCH mediated degradation of LATS1 following activation of the Hippo pathway by ectopic expression of MST2. Whereas ectopic expression of MST2 and LATS1 in HEK293 cells enhanced YAPS127 phosphorylation, coexpression with ITCH led to enhanced degradation of LATS1 and reduced YAPS127 phosphorylation (Fig. 3C). A kinase dead mutant of MST2 failed to enhance LATS1 degradation by ITCH (data not shown). Altogether, these

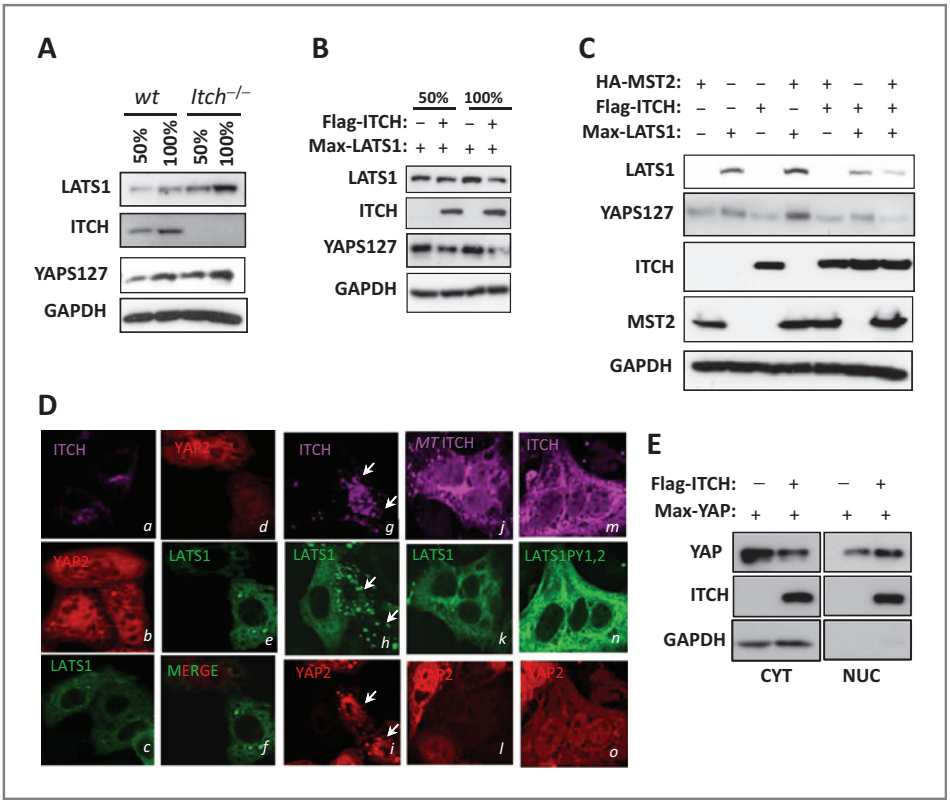

Figure 3. A–C, hippo pathway activation induces ITCH and subsequent LATS1 degradation. A, wild-type MEF and *Itch-/-* cells were cultured at low (50%) and high (100%) cell density in cell culture and cell extract was probed with the indicated antibodies. B, HEK293 cells transfected with the indicated plasmids and cultured at low and high cell density. After 24 hours, cell extract was analyzed as shown in A. C, HEK293 cells were contransfected with the indicated plasmids. After 24 hours, cell extract was analyzed by IB using the indicated antibodies. D and E, ITCH expression is associated with nuclear YAP. D, HeLa cells were cotransfected with either wild-type or mutant EGFP-LATS1 with DsRed-YAP in the presence or absence of either Flag-ITCH or Flag-ITCHC830A. The localization of green fluorescent protein (GFP), Red–tagged, and ITCH was visualized by confocal microscopy using 60x magnification. E, cytoplasmic and nuclear YAP distribution in the presence of absence of ITCH using subcellular fractionation assay and IB as indicated.

results suggest that activation of the Hippo pathway enhances ITCH-mediated degradation of LATS1.

ITCH-mediated degradation of LATS1 leads to accumulation of nuclear YAP

Because YAP localization has been shown to be associated with LATS1-mediated phosphorylation of S127, we next determined whether ITCH might affect YAP subcellular localization. GFP-tagged LATS1 (EGFP-LATS1), pDsRed-YAP2 (Red-YAP2), and Flag-ITCH or mutant Flag-ITCHC830A were coexpressed in HeLa cells and subcellular localization of ITCH, LATS1, and YAP was monitored using confocal microscopy (Fig. 3D, a–c). Co-expression of EGFP-LATS1 and Red-YAP2 showed diffuse and mostly cytoplasmic YAP2 (Fig. 3D, d–f). In contrast, coexpression of ITCH (Fig. 3D, g–i), but not mutant ITCH (Fig. 3D, j–l), enhances the localization of LATS1 into cytoplasmic vesicles and the nuclear localization of YAP2 (arrows). When ITCH was coexpressed with mutant EGFP-LATS1 and Red-YAP2, a stronger LATS1 staining was observed without affecting nuclear YAP localization (Fig. 3D, m–o). To further confirm the effect of ITCH expression on YAP localization we conducted subcellular fractionation of HEK293 cells expressing LATS1 alone or coexpressing ITCH. As shown in Fig. 3E, we observed higher nuclear levels of YAP in the presence of ITCH. These results confirm that ITCH-mediated degradation of LATS1 is accompanied by YAP nuclear localization.

ITCH enhances transcriptional coactivation function of YAP

YAP functions as a coactivator of the TEAD transcription factor (10, 37, 38). Because our data suggest that ITCH overexpression may enhance YAP nuclear localization, we decided to investigate if ITCH can affect YAP transactivation function of TEAD. To this end, we transfecetd *TEAD4-LUC* reporter together with *YAP2* in HeLa cells in the presence or absence of *MST2* and *ITCH*. Under the conditions we used, YAP2 by itself led to 3-fold activation as compared with empty vector (Supplementary Fig. S2B). In contrast, coexpression of YAP with MST2 reduced TEAD transactivation to 1.3-fold. Interestingly, this attenuation was rescued when ITCH was coexpressed with MST2 and YAP. To further confirm the role of ITCH in modulating YAP coactivation function on *TEAD* responsive element, we used the stable *ITCH*-shRNA expressing HeLa clones (Supplementary Fig. S2A). We noticed a significant reduction in reporter activity in these clones compared with either control cells (Supplementary Fig. S2C). Furthermore, when MST2 was coexpressed with YAP, reporter activity was even lower than in parental cells, consistent with higher LATS1 activity and higher phospho-YAP levels (Fig. 2H). To further elucidate that this attenuated YAP activity stems from manipulating LATS1 levels and not as a result of nonspecific ITCH targets, we generated stable HeLa clones that coexpress both *ITCH* and *LATS1* shRNA constructs (Fig. 2H). Analyzing these clones, we noticed that *LATS1* shRNA expression rescued the inhibitory effect of *ITCH* shRNA construct (Supplementary Fig. S2D), confirming that ITCH increases YAP transcriptional coactivation, at least in part, by downregulating LATS1 levels.

ITCH, by targeting LATS1, enhances proliferation, survival, and tumorigenicity

ITCH is a candidate human protooncogene that is overexpressed in different cancer types including anaplastic thyroid carcinoma (39), and in breast cancer, ovarian cancer, and sarcomas as revealed by the Oncomine database, whereas LATS1 has been implicated as a tumor suppressor (20). To explore the potential function of ITCH as an oncogene, we first examined the effect of ITCH depletion on HeLa cell proliferation and survival. Depleting ITCH was associated with reduced proliferation and colony formation (Fig. 4A; Supplementary Fig. S3A–C). Importantly, cells depleted of ITCH and LATS1 exhibited a phenotype resembling control cells (Fig. 4A).

The Hippo pathway determines organ size not only by regulating cellular proliferation but also by inhibiting apoptosis (1). Therefore, we decided to test whether ITCH-dependent degradation of LATS1 affects apoptosis. Previous studies have shown that treatment of HeLa cells with FAS induces apoptosis mediated by MST/LATS association (40); therefore, we assessed the apoptotic sub-G_1 population of ITCH/LATS1 depleted HeLa cells following treatment with anti-FAS antibody (40). We found that depletion of ITCH resulted in increased apoptosis as compared with control cells (Supplementary Fig. S3D). This phenotype was reversed when depleting LATS1 (Supplementary Fig. S3D).

Finally, to test the effect of ITCH on tumorigenesis *in vivo*, we s.c. injected the different HeLa clones into NOD/SCID mice and followed the burden and size of tumors formed. As expected, cells depleted of LATS1 formed bigger tumors, whereas ITCH depleted cells resulted in slowly progressing smaller tumors compared with control cells (Fig. 4B). Moreover, this effect was reversed in cells depleted with ITCH and LATS1 (Fig. 4B).

To further assess the oncogenic function of ITCH, we next tested the effect of ITCH overexpression in normal immortalized human breast epithelial cell-line MCF10A. MCF10A was transduced with lentiviruses expressing ITCH and stable clones were established and used for measuring cell proliferation and colony formation. As shown in Fig. 4C, the MCF10A/ITCH cells proliferated significantly faster than control cells. Furthermore, the MCF10A/ITCH cells showed enhanced cellular transformation and survival as assessed by colony formation and soft agar assays (Fig. 4D and E). Altogether, these results suggest that ITCH-mediated degradation of LATS1 is associated with tumorigenic traits.

ITCH induces expression of YAP target genes related to epithelial-to-mesenchymal transition

Because downregulation of LATS1, but not LATS2, has been shown to phenocopy YAP phenotypes of epithelial-to-mesenchymal transition (EMT; ref. 36), we set to examine whether ITCH-mediated degradation of LATS1 would enhance the upregulation of YAP-associated epithelial-to-mesenchymal transition markers. To this end, we tested the expression levels of known YAP EMT-target genes in the different HeLa cell clones (7, 36). We noticed that while knockdown of LATS1 led to upregulation of these genes, depletion of ITCH led to their downregulation (Fig. 5A). Using double ITCH and LATS1

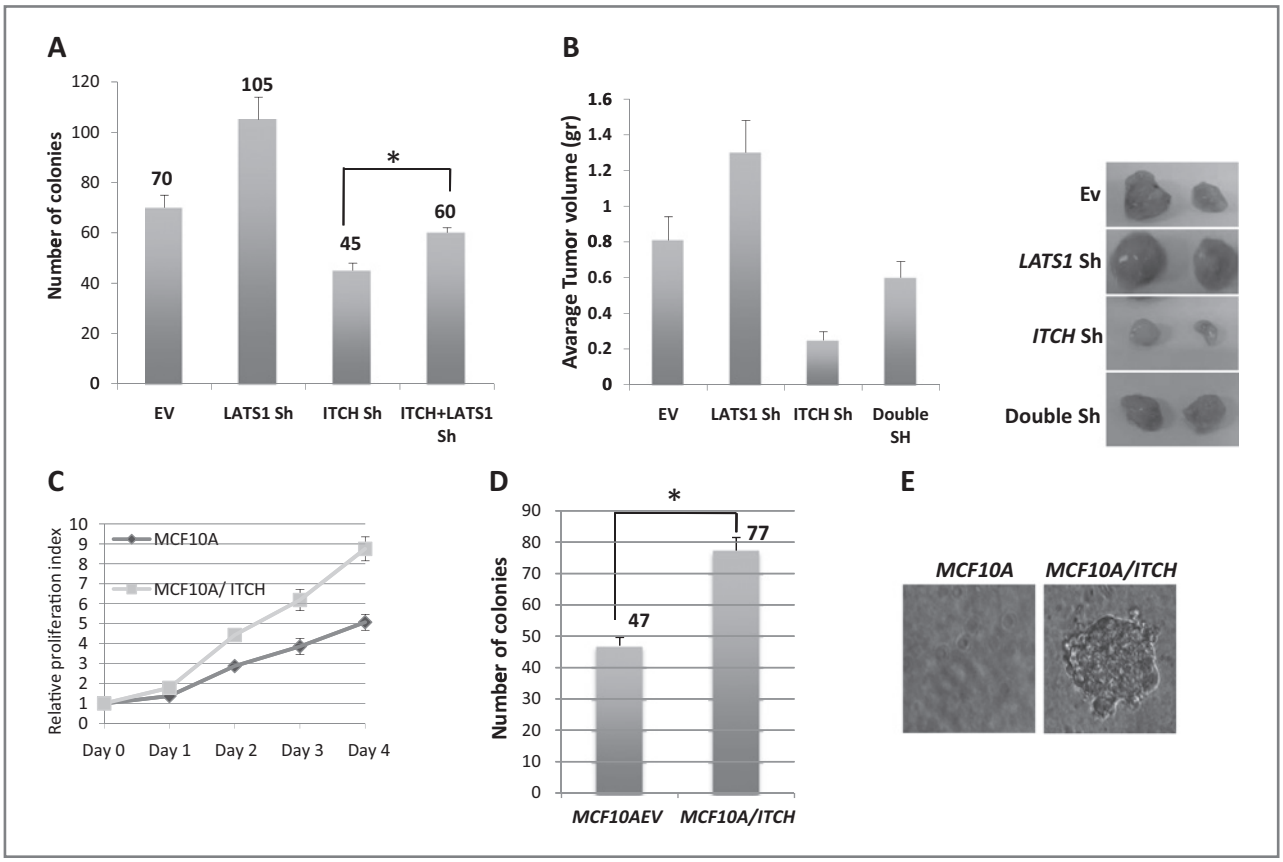

Figure 4. Manipulation of ITCH levels is associated with tumorigenicity. A and B, ITCH knockdown in HeLa cells is associated with A, reduced survival (colony formation assay) and B, reduced tumorigenicity *in vivo*. The indicated HeLa clones were s.c. injected into the flank of NOD/SCID mice. The average tumor weights 40 days postinjection and representative tumor pictures are shown. C, overexpression of ITCH in MCF10A is associated with enhanced proliferation [XTT test (C)], survival [colony formation assay (D)], and transformation [anchorage-independent cell growth (E)]. *, $P < 0.05$.

knockdown expressing cells partly rescued this effect (Fig. 5A). Also, in migration assay we observed that while knockdown of LATS1 in HeLa cells led to more migratory phenotype, depletion of ITCH led to a lesser one (Fig. 5B). Using double ITCH and LATS1 knockdown expressing cells rescued this effect (Fig. 5B). These results suggest that ITCH expression phenocopies YAP overexpression.

These results prompted us to examine whether overexpression of ITCH enhances EMT phenotypes in MCF10A cells. MCF10A vector control cells grew on monolayer cultures and displayed epithelium-type glands (Fig. 6A). ITCH-transduced MCF10A cells, on the other hand, showed an evident loss of epithelium-type characteristics and showed morphology of spindle shape (Fig. 6A). The morphologic alterations were also confirmed by staining for fibronectin and reduced E-cadherin, hallmarks of EMT (Fig. 4S).

To test whether ITCH expression caused EMT of MCF10A cells, Western blotting was carried out to examine the expression levels of several well-characterized EMT markers. Whereas the epithelial marker E-cadherin was downregulated, mesenchymal markers N-cadherin, vimentin, and fibronectin were upregulated in ITCH-overexpressing MCF10A cells (Fig. 6B).

We next carried out wound-healing and Matrigel invasion experiments to examine the activity of ITCH in promoting cell migration and invasion. The motility of ITCH-expressing MCF10A cells was dramatically increased in wound closure and Matrigel invasion (Fig. 6C and D). Finally, we conducted a 3-dimensional culture Matrigel assay. An equal number of cells were plated onto Matrigel in the absence or presence of EGF, and acinar formation was followed at different times after culturing the cells. MCF10A/ITCH cells formed acini in the absence of EGF as early as 3 to 4 days, whereas visible acini were not seen in MCF10A control cells (Fig. 6E). In the presence of EGF, MCF10A cells formed acini after 3 to 4 days and showed lumen formation at 7 to 8 days as expected. By contrast, MCF10A/ITCH cells with EGF formed bigger abnormal spheres that showed spikes and invasive pattern by 6 to 7 days (Fig. 6E and F). Collectively, these data show that ITCH promotes the EMT of MCF10A cells, possibly by regulating LATS1-YAP function and in turn the expression of key transcription factors involved in EMT.

Discussion

In this study, we have shown that the ubiquitin E3 ligase ITCH physically and functionally associates with LATS1. Three lines of evidence suggest that ITCH is a physiologic regulator of LATS1. First, overexpression of ITCH effectively ubiquitinated

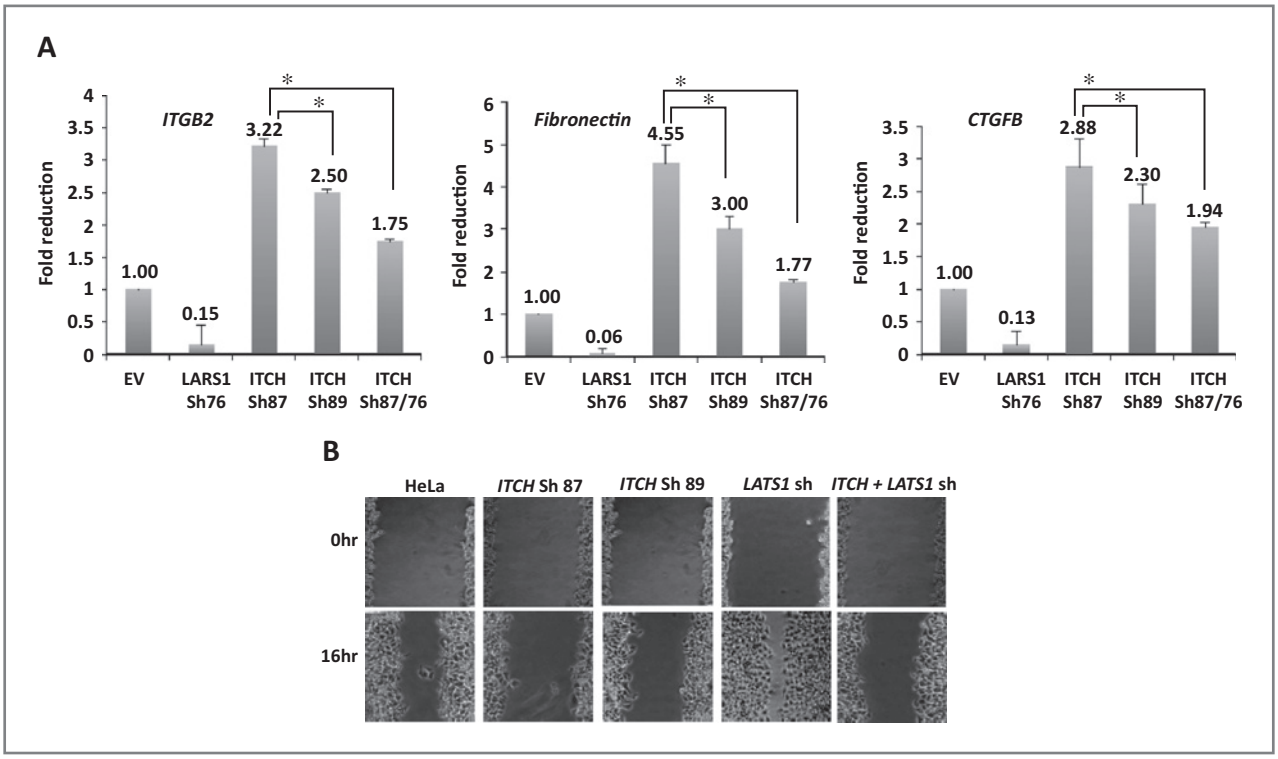

Figure 5. Effect of ITCH knockdown on EMT in HeLa cells. A, Real-time PCR analysis for *ITG*β*2*, *Fibronectin*, and *CTGF*β in the different HeLa clones. GAPDH was used for normalization. B, wound healing assay showing the migration of the indicated HeLa clones *, $P < 0.05$.

and degraded LATS1. A point mutation in the PPxY motifs of LATS1 or enzymatic inactive ITCH failed to degrade LATS1. Second, knockdown of ITCH effectively phenocopied LATS1 overexpression, such as reduced cell proliferation and tumorigenicity and enhanced apoptosis. Third, the phenotypes associated with ITCH knockdown could be suppressed by the simultaneous knockdown of LATS1. Although we cannot exclude that other E3 ligases target LATS1, ITCH seems to regulate LATS1 protein degradation through WW/PPxY-specific association. Our findings may also suggest that different WW domain-containing proteins compete for interacting with LATS1 hence determining its function. Nevertheless, our findings provide further evidence of WW domain-containing proteins regulating the function of the Hippo pathway.

Upon activation by stimuli such as high cell density in cell culture, the Hippo pathway phosphorylates and inactivates YAP, an outcome that represents the major signaling output of the pathway (1, 2). Our findings provide evidence that activation of the Hippo pathway is associated with increased levels of ITCH. These results may represent a fine-tuning mechanism of the Hippo pathway regulation through which the level of LATS1 is posttranslationally regulated. Intriguingly, culturing MEFs under high-density conditions was associated with slight increase in LATS1 levels and enhanced YAPS127 phosphoryaltion which was further evident in *Itch*-deficient cells suggesting that ITCH deficiency impairs regulation of LATS1 levels. These results are consistent with our observation that the activation of the Hippo pathway results in a prominent LATS1-ITCH interaction, and previous published data highlighting the differential regulation of the Hippo pathway in different contexts (9, 41). Our results may also suggest that in absence of ITCH, other, perhaps WW domain-containing, proteins could stabilize LATS1.

Recently, it has been shown that LATS1-mediated phosphorylation of YAP on Ser381 primes YAP for subsequent phosphorylation by CKIδ/ε in a phoshodegron manner leading to YAP degradation mediated by SCF-$^{(\beta TCRP)}$ E3 ubiquitin ligase, thereby suppressing YAP oncogenic potential (41). Our results suggest that ITCH catalyzing ubiqutination of LATS1 leads to reduced YAP phosphorylation and perhaps YAP stabilization thus promoting its oncogenic potential. Indeed, we show that ITCH-mediated LATS1 degradation is associated with increased cell proliferation, survival, migration, tumorigenesis *in vivo,* and resistance to FAS-induced apoptosis. Of note, these phenotypes were rescued, at least in part, by depletion of LATS1. These findings suggest that LATS1 is a critical substrate of ITCH mediating tumor growth and progression.

Because ITCH regulates the stability of several substrates, including p73 and p63 (42), we cannot exclude the possibility that other proteins could also contribute to the phenotypes observed in our study. For example, ITCH could degrade p73 (30) and promote active YAP localization to the nucleus, fueling its oncogenic potential through TEAD. Because YAP is an important cofactor for p73-dependent transcriptional activity and exerts a tumor suppressor role in this context (43, 44), ITCH overexpression might serve as a molecular switch between opposing YAP functions (45). Whether YAP relocates between p73/YAP targets and TEAD/YAP targets in response

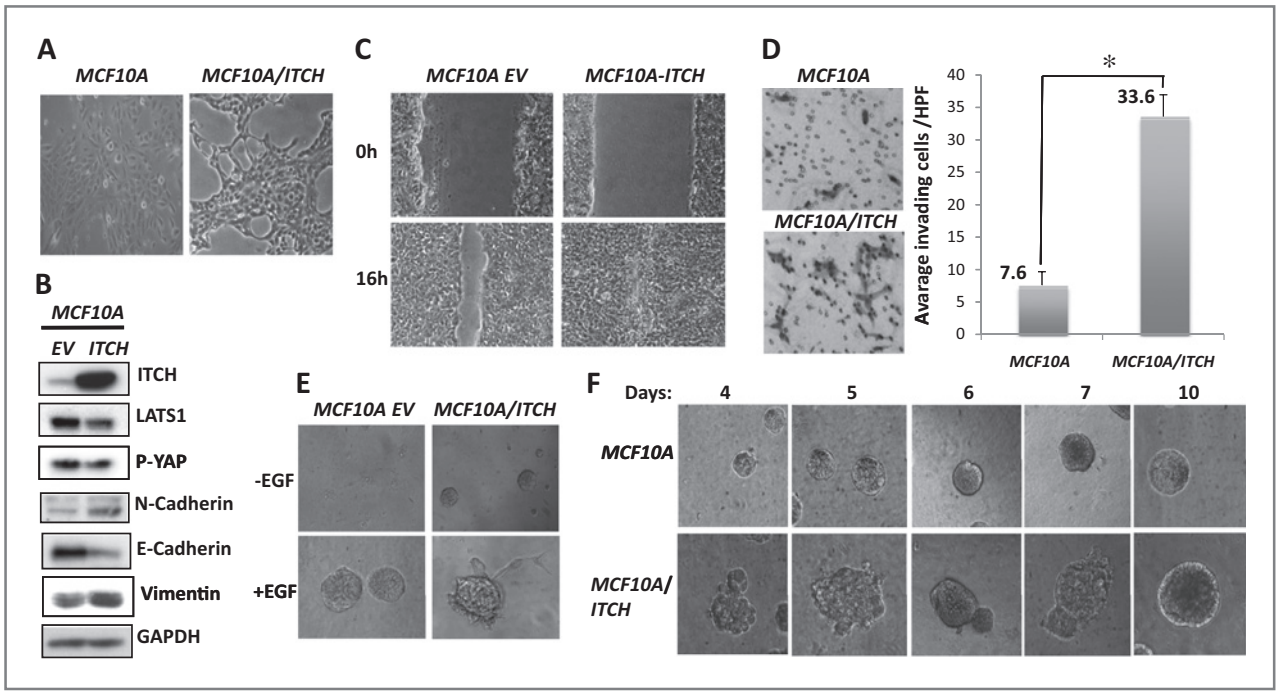

Figure 6. ITCH overexpression correlates with EMT in MCF10A cells. A, morphology of MCF10A and MCF10A/*ITCH* in 2-dimensional culture. B, IB analysis showing the effect of ITCH overexpression in MCF10A on the indicated proteins. C, migration of MCF10A stable clones into the wound was monitored as indicated in absence of EGF. D, Matrigel-invasion assay of MCF10A clones in presence of EGF. Bars show quantification of number of invading cells of 10 fields. E, mammary spheroid formation in MCF10A clones in the absence and presence of EGF at 3–4 days. F, effect of ITCH in MCF10A cells on spheroid formation in 3-dimensional Matrigel culture. Spheroid formation progression is shown on different days.

to ITCH is to be determined in future studies. Although ITCH is emerging as a prosurvival factor, ITCH may also catalyze degradation of prooncogeneic factors, such as the Gli transcription factor that is deregulated in medulloblastomas (46). Further investigation of ITCH phenotypes in animal models is required to uncover its role in tumorogenesis and in different contexts.

Results from several independent assays suggested that ITCH phenocopies YAP overexpression in the Hippo pathway. First, expression of ITCH leads to reduction of LATS1 levels and reduced YAP phosphorylation. Second, ITCH overexpression resulted in increased cell migration of MCF10A cells, conferred growth ability to MCF10A cells, and abnormal spheroid structure in 3-dimensional culture. On the other hand, depleting ITCH in HeLa cells is associated with reduced tumorigenicity. Collectively, these results strongly argue that ITCH overexpression phenocopies phenotypes related to YAP overexpression and suggest that ITCH may be a critical endogenous negative regulator of LATS1 in mammalian cells. The induction of EMT by ITCH overexpression is consistent with an emerging concept of EMT inducers as oncogenes. In fact, ITCH is amplified and/or upregulated in several types of human malignancies (39). The observation that ITCH overexpression alone is sufficient for EMT suggests that it controls the mammalian Hippo pathway.

LATS1 is a multifunctional protein involved in diverse cellular processes, including cell cycle, cell proliferation, and apoptosis (20). The identification of ITCH as the E3 ligase for ubiquitination and degradation of LATS1 also sheds new light on the roles of these proteins in cancer. Although it is reported that promoter hypermethylation and mutation may be one possible mechanism in the downregulation of LATS1 in human cancer (20), it is possible that an increase in ITCH expression can also play a part in LATS1 down-regulation. Screening of ITCH and LATS1 expression in human cancer should therefore shed light on this possible inverse correlation. Most recently, it was shown that the heat shock protein 90 (HSP90) interacts with LATS1/2 and regulates their levels (47). Functionally, HSP90 inhibitors deplete LATS1/2, reduce YAP phosphorylation, and increase levels of CTGF, a gene that is coactivated by YAP. In agreement with our results, these findings suggest that disrupting the LATS tumor suppressor pathway in human cancer cells is associated with inactivating the Hippo pathway and tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by grants from Israeli Science Foundation (#1331–08) to R.I. Aqeilan.

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Received September 29, 2010; revised December 16, 2010; accepted December 21, 2010; published OnlineFirst January 6, 2011.

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