THE UBIQUITIN LIGASE ITCH REGULATES APOPTOSIS BY TARGETING THIOREDOXIN-INTERACTING PROTEIN FOR UBIQUITIN-DEPENDENT DEGRADATION

Pingzhao Zhang‡§1, Chenji Wang‡1, Kun Gao§, Dejie Wang§, Jun Mao§, Jian An‡, Chen Xu‡, Di Wu‡, Hongxiu Yu§, Jun O. Liu‡ and Long Yu‡§2*

Runing head: Itch ubiquitinates and degrades TXNIP

From the ‡State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, 220 Handan Road, Shanghai 200433, P.R. China, the §Institutes of Biomedical Sciences, Fudan University, Shanghai, 200032, P. R. China, the ¶Key Laboratory of Transplant Engineering and Immunology, Ministry of Health, West China Hospital, Sichuan University, Chengdu, 610041, P.R. China, and the ‡§Departments of Pharmacology and Molecular Sciences and oncology, The Johns Hopkins School of Medicine, Baltimore, MD 21205

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed: State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, 220 Handan Road, Shanghai 200433, P.R. China. Tel: +86-21-65643954; Fax: +86-21-65643250; E-mail: longyu@fudan.edu.cn.

Thioredoxin interacting protein (TXNIP) was originally characterized as an endogenous inhibitor of thioredoxin, a key regulator in cellular redox homeostasis. TXNIP is also known to play important roles in tumor growth and metastasis, glucose and lipid metabolism. TXNIP expression is induced by various stress stimuli. However, it has been unclear how TXNIP is downregulated. Here, we report that TXNIP undergoes proteasomal degradation in cells. We identify Itch as the E3 ubiquitin ligase for TXNIP. We demonstrate that Itch mediates poly-ubiquitination of TXNIP both in vitro and in vivo. Overexpression of Itch leads to TXNIP proteasomal degradation. Knockdown of Itch by small interfering RNA causes an accumulation of steady-state level of TXNIP. We also show that the PPXY motifs of TXNIP and the WW domains of Itch mediate their interaction. Furthermore, the Itch-TXNIP interaction regulates intracellular ROS levels and apoptosis. These findings establish a new mechanism for the negative regulation of TXNIP by Itch and shed new light on the regulation of cellular redox homeostasis.

The intracellular redox homeostasis is maintained in part by the reactive oxygen species (ROS)-scavenging system, an important component of which is thioredoxin. Thioredoxin reduces ROS through its free thiols at two cysteine residues (Cys-32 and Cys-35). And oxidized thioredoxin is recycled to its reduced state by thioredoxin reductase and NAPDH (1).

Thioredoxin-interacting protein (TXNIP), an endogenous inhibitor of thioredoxin also known as vitamin D₃ up-regulated protein-1 (VDUP1) or thioredoxin-binding protein-2 (TBP-2),
inhibits antioxidative function of thioredoxin by binding to its redox-active cysteine residues (1-3). By negatively regulating thioredoxin, TXNIP is involved in a wide variety of cellular processes such as cell proliferation or apoptosis (4).

TXNIP has also been shown to be an important tumor suppressor, and its expression is dramatically reduced in various types of human tumors (5,6). Overexpression of TXNIP inhibits cell proliferation and promotes apoptosis. Point mutation or knockout of the TXNIP gene in a mouse model is associated with a higher incidence of hepatocellular carcinoma (7). Furthermore, increased TXNIP expression inhibited melanoma metastasis and up-regulated KISS1, suggesting TXNIP is also a metastasis suppressor (5).

It has been shown that the Krebs cycle-mediated fatty acid utilization was impaired in TXNIP knockout mice, indicating its involvement in lipid metabolism (8). Furthermore, TXNIP is a critical mediator of glucose-induced beta cell apoptosis (9). TXNIP null mice have fasting hypoglycemia with a striking enhancement of glucose uptake by peripheral tissues (10-12). In humans, TXNIP expression is suppressed by insulin and strongly upregulated in diabetes, suggesting that TXNIP is a critical regulator of glucose metabolism in vivo (13).

Protein ubiquitination has emerged as a fundamental mechanism for regulating the half lives and activity of many cellular proteins. The specificity of ubiquitination reaction is achieved by the E3 ubiquitin ligases (E3’s), which mediate the transfer of ubiquitin from E2 ubiquitin-conjugating enzymes (E2) to substrates. Ubiquitination controls turnover and abundance of proteins by targeting them for proteasomal degradation (14). The Nedd4-like family of E3 ubiquitin ligases is characterized by a 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 coordinating with the E2 and providing the catalytic E3 activity (15-17). The Nedd4 family contains nine members in human including Nedd4, Nedd4-2, Itch, Smurf1, Smurf1, WWP1, WWP2, NedL1, and NedL2 (17). The E3 ubiquitin ligase Itch was originally identified as a gene disrupted in the non-agouti-lethal 18H mice, or Itchy mice that suffer from severe immune and inflammatory defects. A number of Itch proteolysis targets are central players in or regulators of multiple signaling pathways, including c-Jun, JunB, p73, p63, C-FLIP and others (18).

Herein, we report that the TXNIP is negatively regulated by Itch in cancer cells. We demonstrate that Itch directly interacts with and acts as a robust E3 ubiquitin ligase for TXNIP. Overexpression of Itch promotes ubiquitination and proteasomal degradation of TXNIP. Conversely, knockdown of Itch by siRNAs increases TXNIP steady-state level. Furthermore, Itch may modulate ROS-induced apoptosis by controlling TXNIP protein level.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and transfection**—293T, H1299 and U2OS cells were obtained from the American Type Culture Collection. 293T cells were maintained in DMEM with 10% FBS. H1299 cells were maintained in RPMI 1640 with 10% FBS. U2OS cells were maintained in McCoy's 5A medium with 10% FBS. Cells were transiently
transfected using Lipofectamine (Invitrogen, USA) according to manufacturer’s instructions.

**Expression constructs**—Human Flag-TXNIP plasmid was kindly provided by Dr. Junji Yodoi (Kyoto University, Japan) and subcloned into pCMV-HA (Clontech, USA) and pGEX-4T-2 vectors to add HA and GST tags, respectively. Wild type and catalytically inactive mutant (C830A) Myc-Itch were kindly provided by Dr. Gerry Melino (Leicester University, UK). GFP-Itch and GST-Itch were kindly provided by Dr. Annie Angers (Université de Montréal, Canada). Myc-Smurf1/2 and Myc-WWP1 were kindly provided by Dr. Kohei Miyazono (University of Tokyo, Japan). HA-Nedd4 was kindly provided by Dr. Xuejun Jiang (Memorial Sloan-Kettering Cancer Center, USA) and subcloned into pCMV-Myc vector. All other TXNIP or Itch mutants were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene, USA).

**RNA Interference**—The RNAi oligos for Itch and TXNIP were purchased from Genepharma (Shanghai, China). The RNAi oligos sequences for Itch are:

- **RNAi #1:** 5′-CCAGUUGGACUCAAGGAUUAdTdT-3′;
- **RNAi #2:** 5′-GGUGACAAAGAGCCAACAGAGdTdT-3′.

The sequence of negative control is:

- **Control RNAi:** 5′-ACAGACUUCGGAGUACCUGdTdT-3′.

**Quantitative RT-PCR**

Total RNA was isolated from U2OS cells using the TRIZol reagent (Tiangen, China), and cDNA was reversed-transcribed using the Superscript RT kit (TOYOBO, Japan), according to the manufacturer’s instructions. PCR primer sequences for human TXNIP were selected as follows:

- **AGTTACTCGTGTTCAAGGCGGCCTAG(forward)**
- **TCACCATCTCATTCACCTCTGAGT(reverse)**

PCR amplification was performed using the SYBR Green PCR master mix Kit (TOYOBO, Japan). All quantitations were normalized to the level of endogenous control GAPDH.

**Antibodies**—For Western blot, the following antibodies were used: mouse monoclonal antibodies against TXNIP (JY2; MBL), Itch (sc-28367; Santa Cruz, USA), Myc (9E10; Sigma, USA), FLAG (M2; Sigma), HA (MM5-101R; Convance, USA), GFP (sc-8334; Santa Cruz, USA), Actin (AC-74; Sigma, USA), GST (710974; Novagen, USA), Ubiquitin (U5379; Bethyl, A300-317A, USA).

**Immunoprecipitation**—Cells were lysed with cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM EGTA, 1 mM Na2EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4 and 1 µg/ml leupeptin) and the
lysate was centrifuged. The supernatant was precleared with protein A/G beads (Sigma, USA), followed by incubation with 2 µl antibody for 2 hours and thereafter with protein A/G beads for 2 hours, all at 4°C. Pellets were washed 4 times with lysis buffer and resuspended in sample buffer and analyzed by SDS–PAGE.

**Western blot**- Cell lysates and immunoprecipitates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes (GE Healthcare, USA). The membrane was blocked in PBS containing 5% non-fat milk and 0.1% Tween-20, washed twice in PBS, and incubated with primary antibody at room temperature for 2 hours, followed by incubation with secondary antibody at room temperature for 45 minutes. Afterward, the proteins of interest were visualized using ECL chemiluminescence system (Santa Cruz Biotechnology, USA).

**GST-pulldown assay**- HEK 293 T cells were lysed 36 h after transfection with cell lysis buffer for 30 min at 4°C. GST fusion proteins were immobilized on glutathione-Sepharose beads (Amersham Biosciences). After washing with pull-down buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 10% glycerol, 1 mM EDTA, 2.5 mM MgCl₂ and 1 µg/ml leupeptin), the beads were incubated with lysates of transfected H1299 cells for 4 h. The beads were then washed four times with binding buffer and resuspended in sample buffer. The bound proteins were subjected to SDS–PAGE.

**In vivo and in vitro ubiquitination assays**- The in vivo ubiquitination assay was conducted as previously described (19). In vitro ubiquitination assay was carried out in a buffer containing 50 mM HEPES (pH 7.9), 5 mM MgCl₂, 15 µM ZnCl₂, and 4 mM ATP, with 100 nM E1 (Sigma, USA), 200 nM human recombinant UbcH7, 250 µM ubiquitin (Sigma, USA). In vitro reactions were carried out at 37°C for 60–90 min.

**Immunofluorescence**- COS7 cells cultured on coverslips were fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100 for 5 min at room temperature. The coverslips were blocked with 5% normal goat serum plus 2% BSA for 1 h and then incubated with mouse anti-HA antibody (1:300 dilution) for 1 h at room temperature, which was followed by sequential incubation with a Texas Red-conjugated goat anti-mouse secondary antibody at 1:300 dilution, and with DAPI (1:500 dilution) for 10 min. Epifluorescence images were captured using Olympus Inverted System Microscope.

**ROS assay**- U2OS cells were seeded overnight in 6-well plates. Forty eight hours after transfection, cells were trypsinized and centrifuged, followed by incubation in 5 µM H2-DCF-DA for 30 min at 37°C. Cells were washed twice, suspended in PBS, and analyzed immediately by FACScan (Becton-Dickinson) that was equipped with a 488 Argon laser for measurements of intracellular fluorescence. Logarithmic detectors were used for the FL-1 fluorescence channel necessary for DCF detection. Mean log fluorescence intensity (MFI) values were obtained by the CELLQUEST software program.

**Apoptosis Assay**- U2OS cells were seeded overnight in 6-well plates. Forty eight hours after transfectoin, cells were treated with 20 µM etoposide for 12 h. The cells were collected and fixed with 2 ml of 70%
ethanol at 4°C for 2 h. Cells were washed with PBS and incubated in PBS containing 100 μg/ml RNase A and 50 μg/ml propidium iodide (PI) for 15 min at room temperature. DNA content and cell cycle were assessed by FACScan. Based on propidium iodide staining, cells in sub-G1 were considered apoptotic.

Statistical analysis- The ROS and apoptosis data in this study were expressed as the mean ± S.D. from at least three independent experiments. Statistical analysis was performed using one-way ANOVA with a Newman-Keuls post-hoc test. A value of p < 0.05 was considered statistically significant.

RESULTS
TXNIP stability is controlled by the ubiquitin-proteasome pathway- Although it is known TXNIP mRNA is rapidly induced by various stresses stimuli (4), little is known about the post-translational regulation of TXNIP. Given its relatively rapid turnover, we decided to investigate whether TXNIP was subjected to ubiquitination-dependent degradation. Since most cellular protein degradation is mediated by the proteasomal pathway, we treated U2OS and 293T cells with the proteasome inhibitor MG132 for 0, 0.5, 1 and 2 h and determined the protein levels of endogenous TXNIP by Western blot. As shown in Fig.1A (upper and middle panels), MG132 treatment led to a rapid increase in TXNIP protein level than that caused by MG132 in U2OS cells. To exclude the possibility that higher protein level might have resulted from upregulation of transcription, we performed qRT-PCR to measure the mRNA level of TXNIP in U2OS cells upon lactacystin treatment. As shown in Fig. 1B, lactacystin has little effect on the mRNA level of TXNIP. Together, these results suggested that TXNIP stability might be regulated by the proteasomal pathway.

As proteins destined to proteasomal degradation are often ubiquitinated, we next determined whether TXNIP underwent ubiquitination in vivo. First, we co-expressed HA-TXNIP and (His)_6-ubiquitin in H1299 cells. TXNIP conjugated to (His)_6-ubiquitin was pulled down using Ni^{2+}-NTA beads under denaturing conditions and detected by Western blot with the TXNIP-specific monoclonal antibody JY2. As shown in Fig. 1C (left panel), TXNIP did undergo ubiquitination in vivo. Second, we transiently expressed flag-TXNIP in H1299 cells. Upon MG132 treatment for 4 h, cells were lysed and the Flag-TXNIP protein was immunoprecipitated. The poly-ubiquitinated forms of TXNIP were detected by Western blot with anti-Ub antibody. As shown in Fig. 1C (right panel), inhibition of the proteasome by MG132 caused accumulation of the poly-ubiquitinated forms of TXNIP.

The type of ubiquitin linkage determines the fate of ubiquitinated proteins. K48-linked ubiquitination generally directs proteins to the proteasome for degradation, whereas K63-linked ubiquitination usually affects protein function or protein-protein interactions (21). To determine which type of ubiquitin linkage is involved in the ubiquitination of TXNIP, we examined the
ability of HA-tagged wild-type ubiquitin (WT) or ubiquitin mutants to ubiquitinate TXNIP. The K48R and K63R ubiquitin mutants containing a single lysine to arginine mutation at position 48 and 63 are expected to disrupt K48 and K63 ubiquitin linkage. On the other hand, K48 only and K63 only ubiquitin mutants contain arginine substitutions on all lysine residues except the one at position 48 and 63, respectively, and are thus expected to promote the proteasome-linked K48 and the proteasome-independent K63 ubiquitin linkages, respectively. The K0 ubiquitin mutant is a lysineless ubiquitin mutant capable of mediating mono-ubiquitination only. Flag-TXNIP and various ubiquitin mutants were co-expressed in H1299 cells. TXNIP were immunoprecipitated and its patterns of ubiquitination were determined by Western blot with anti-Ub anitbody. As shown in Fig. 1D, TXNIP ubiquitination was largely abolished when it was co-expressed with Ub-K0 mutant, suggesting that TXNIP cannot be monoubiquitinated at multiple sites. Interestingly, TXNIP ubiquitination was also greatly abolished when it was co-expressed with Ub-K48 only or Ub-K63 only, but was similar when it was co-expressed with Ub-K48R and Ub-K63R, respectively. It is possible that TXNIP polyubiquitination was mediated by other unusual ubiquitin linkage, such as K6, K11, K27 and K29. It has been reported that all these non-K63 linkages are abundant in vivo and may also target proteins for degradation (22). Another possibility is that the ubiquitin linkage of TXNIP is heterogeneous and dynamic in vivo. The precise mode of polyubiquitination of TXNIP remains to be elucidated.

E3 ligase Itch promotes TXNIP degradation- Next, we searched for the relevant E3 ligase that was responsible for TXNIP degradation. Sequence alignment of multiple TXNIP orthologs across different species revealed that two PPXY motifs at the C-terminal region of TXNIP are highly conserved (Fig. 2A). PPXY motif is known to interact with WW domain-containing proteins, which includes Nedd4-like family of E3 ubiquitin ligases. This family of proteins are typically comprised of a catalytic C-terminal HECT domain and N-terminal C2 domain and WW domains. The WW domains mediate ligase-substrate recognition through interactions with the PPXY consensus sequence (16). Thus, we determined if one or more member of Nedd4-like family of E3 ubiquitin ligases might interact with the conserved PPXY motifs present in TXNIP and mediate its ubiquitination. HA-TXNIP was co-expressed with a panel of Nedd4 family of E3 ubiquitin ligases, including Itch, Smurf1, Smurf2, WWP1 and Nedd4, in H1299 cells. As shown in Fig. 2B, of all E3 ubiquitin ligases tested, only Itch efficiently promoted TXNIP degradation in a dose-dependent manner. These results raised the possibility that the polyubiquitination of TXNIP may be mediated by Itch.

Itch is a key regulator of TXNIP stability- To verify the role of Itch in TXNIP degradation, we tested an Itch mutant in which the highly conserved Cys residue (C830) in its HECT domain was mutated to Ala (Itch/C830A). The Cys830Ala mutation has been shown to abrogate Itch’s ubiquitin ligase activity (23). As shown in Fig. 3A, wild type Itch, but not the catalytically inactive Itch mutant, promoted TXNIP degradation in a dose-dependent manner, indicating that the HECT domain of Itch and its ubiquitin ligase activity are required for promoting TXNIP degradation.
Consistent with this observation, TXNIP degradation induced by Itch co-expression could be completely rescued by the proteasome inhibitor MG132 (Fig. 3B). In the absence of co-transfected Itch, TXNIP was relatively stable and did not accumulate in the presence of MG132.

We also determined the effects of Itch and the Itch/C830A mutant on TXNIP protein turnover. H1299 cells were co-transfected with HA-TXNIP expression plasmid and empty vector, Itch or Itch/C830A mutant. After 24 h, the cells were treated with cycloheximide (CHX) to block protein synthesis. As shown in Fig. 3C and 3D, co-expression of wild type Itch and TXNIP resulted in a striking decrease in TXNIP protein level, indicating that Itch promoted TXNIP degradation. In contrast, TXNIP was stabilized by the Itch/C830A mutant, likely due to a dominant negative effect of the catalytically inactive mutant on the wild type enzyme.

Next, we overexpressed wild type Itch and the Itch/C830A mutant in 293T cells and determined their effects on the protein level of endogenous TXNIP. As shown in Fig. 3E, overexpression of wild type Itch led to a significant reduction in endogenous TXNIP. In comparison, the Itch/C830A mutant had no effect on endogenous TXNIP level. Similar results were obtained in U2OS cells (data not shown). In a complementary experiment, we knocked down the endogenous Itch by two specific siRNAs and determined the changes in TXNIP protein level in 293T and U2OS cells, respectively. Knockdown of Itch resulted in an increase in the level of endogenous TXNIP (Fig. 3F). To exclude the possibility that TXNIP protein elevation resulted from transcriptional upregulation, we performed qRT-PCR to measure the mRNA level of TXNIP and Itch in siRNA knockdown U2OS cells. In contrast to over 4-fold decrease in Itch mRNA transcripts, the mRNA level of TXNIP in Itch depleted U2OS cells stayed at a level similar to that of control cells (Fig. 3G). Taken together, these results further support the notion that Itch is a key regulator of TXNIP stability.

TXNIP forms a complex with Itch- Given that Itch contains WW domains that are known to mediate its binding to substrates through PPXY motif, which were also present in TXNIP, we examined the interaction between these two proteins. We co-expressed HA-TXNIP and wild type Itch or the Itch/C830A mutant (which was used to avoid degradation of the protein bound to Itch) in H1299 cells and immunoprecipitated TXNIP. As shown in Fig. 4A, Itch coimmunoprecipitated with TXNIP. This interaction was independent of ubiquitin ligase activity of Itch, as Itch/C830A mutant also coimmunoprecipitated with TXNIP. Similar results were obtained in a reciprocal coimmunoprecipitation experiment using anti-Myc antibody (Fig. 4B). Furthermore, when endogenous TXNIP was immunoprecipitated by anti-TXNIP antibody, endogenous Itch was detectable in the immunoprecipitate by Western blot (Fig. 4C).

To verify the interaction between TXNIP and Itch in vivo, we investigated whether these two proteins are localized to the same subcellular compartments. The subcellular localization of TXNIP and Itch in COS-7 cells has been reported previously. Itch was found in the trans-Golgi network and endosomal compartments (24), while TXNIP was mainly localized to the nucleus (25). HA-TXNIP and GFP-Itch expression constructs were transfected into COS7 cells, respectively. Their subcellular
localizations were similar to those previously reported (Fig. 4D). But when HA-TXNIP and GFP-Itch were co-expressed in COS-7 cells, a significant proportion of HA-TXNIP was co-localized with GFP-Itch at small speckle structures in cytoplasm, which were reminiscent of endosomes (Fig. 4D). Since TXNIP was known to interact with Importin α1 and to be imported into the nucleus to exert its physiological effects including growth-suppressive activity, this result suggested that TXNIP could be sequestered in the cytoplasm where it is degraded through Itch.

**Binding of Itch to TXNIP is mediated through the WW domain of Itch and the PPXY motif of TXNIP.** TXNIP contains two arrestin domains and a C-terminal domain with two PPXY motifs (331aa ~ 334aa:PPCY; 375aa ~ 378aa:PPPY). Itch contains multiple WW domains at its central region. To determine whether the Itch-TXNIP interaction is mediated through these domains, we generated truncation mutants of TXNIP in which one (ΔPY1 and ΔPY2) or both (ΔPY1/2) PPXY motifs are deleted or a C-terminal (which harbor the second PPXY motif) deletion mutant (ΔC). We also constructed a single amino acid mutant (PYF) by substituting the terminal Tyr (Y) of the second PPXY for Phe (F). These mutations have been shown to abrogate binding of PPXY motif to WW domains. All five mutants, as well as TXNIP WT (Wild Type), were fused to GST and expressed in and purified from bacteria. We used a pull-down assay with GST fusion proteins of TXNIP and Itch overexpressed in H1299 cells. As shown in Fig. 5A, wild type and ΔPY1 TXNIP interacted with Itch. But the interactions were nearly abolished between Itch and other TXNIP mutants, including ΔPY2, ΔPY1/2, ΔC and PYF. Thus, the second PPXY motif appeared to be indispensable for the interaction of TXNIP with Itch.

Next, we generated three deletion mutants of Itch and determined the minimal domain of Itch that is sufficient to mediate its interaction with TXNIP. Thus, Wild type (WT) and three Itch deletion mutants containing the C2 domain (C2), WW domain (WW), HECT domain (HECT), respectively, were fused to GST and expressed in and purified from bacteria. A GST pull-down assay for Itch and HA-TXNIP expressed in H1299 cells was carried out. As shown in Fig. 5B, TXNIP interacted with the WW mutant harboring four WW domains. Thus, Itch is capable of interacting with TXNIP through multiple WW domains.

We also determined if the PPXY motifs were essential for Itch-mediated degradation as well. As shown in Fig. 5C, Itch targeted wild type TXNIP for degradation efficiently, but not the ΔPY2, ΔPY1/2, ΔC, or the PYF mutant. Surprisingly, Itch was also unable to target TXNIP ΔPY1 mutant for degradation, even though this mutant retained the ability to interact with Itch (Fig. 5A). These results suggested that Itch interacts with both two PPXY motifs of TXNIP through its multiple WW domains. Although the second PPXY motif is dispensable for Itch-TXNIP interaction in GST pull down assay, it is still required for the degradation of TXNIP by Itch in vivo.

**Itch ubiquitinates TXNIP both in vitro and in vivo.** Given that Itch is capable of binding to TXNIP and inducing its degradation upon coexpression, it is highly likely that TXNIP is a ubiquitination substrate of Itch. To assess this possibility, we co-expressed...
Flag-TXNIP, HA-Ubiquitin and Itch, or Itch/C830A mutant in H1299 cells. The polyubiquitinated forms of TXNIP were immunoprecipitated and then detected by Western blot with anti-Ub antibody. As shown in Fig. 6A (see short exposure), TXNIP was seen as a strong smear of bands when it was co-expressed with wild type Itch. We also employed an in vitro ubiquitination assay using purified recombinant proteins to determine whether TXNIP is a direct ubiquitination substrate for Itch. As shown in Fig. 6B, Itch caused poly-ubiquitination of TXNIP in the presence of E1, E2 UbcH7 and (His)_6-Ub. In contrast, the Itch/C830A mutant failed to cause TXNIP poly-ubiquitination. These results indicated that TXNIP is a direct substrate of Itch in vitro and likely in vivo.

Itch modulates basal level of intracellular ROS by controlling TXNIP protein level- It has been shown that TXNIP is an inhibitor of thioredoxin, which possesses anti-apoptotic activity by scavenging ROS (1). TXNIP is induced by various apoptosis-inducing stimuli and associated with increasing levels of ROS through reduction of thioredoxin activity (2). Since Itch controls steady-state level of endogenous TXNIP, we assessed whether Itch had any effect on the intracellular ROS level. As expected, knockdown of TXNIP decreased intracellular ROS level while knockdown of Itch increased intracellular ROS level (Fig. 7A). Notably, concomitant knockdown of TXNIP largely reversed the positive effect of Itch knockdown on intracellular ROS level (Fig. 7B). These results suggested that Itch may play a role in regulating intracellular ROS Levels at least in part, by regulation of TXNIP protein stability.

Knockdown of Itch promotes etoposide-induced ROS accumulation and apoptosis- TXNIP has been implicated in the regulation of apoptosis induced by various stress stimuli by increasing levels of ROS. As the chemotherapeutic drug etoposide has been reported to cause significant ROS accumulation and induce apoptosis (26), we determined if Itch had an effect on ROS accumulation and apoptosis induced by etoposide. As shown in Fig. 8A, knockdown of Itch in U2OS cells increased intracellular ROS level upon treatment with etoposide. The increase in ROS level was accompanied by an increase in apoptosis (Fig. 8B). These observations suggest that Itch may modulate chemotherapy drug-induced ROS accumulation and apoptosis in U2OS cells.

DISCUSSION

TXNIP is a multifunctional protein involved in diverse cellular processes, including cell proliferation, apoptosis and differentiation (2,4). TXNIP expression is under tight control in normal cells and deregulation of TXNIP has been implicated in cancer, cardiac and metabolic diseases (1,4). TXNIP is induced by various stress stimuli, including H_2O_2, irradiation, UV, heat shock, serum deprivation, and growth-inhibitory factor such as transforming growth factor-β1 (4). Anticancer agents such as 5-fluorouracil, anisomycin, dexamethasone, and ceramide also dramatically induce TXNIP expression (27). Much is known about the transcriptional regulation of TXNIP expression. A number of transcriptional factors, including Heat shock factor, glucocorticoid receptor, MondoA, FOXO1, have been identified that regulate TXNIP expression under different conditions.
In contrast, the post-transcriptional and post-translational regulation of TXNIP has remained largely unknown. Recently, some hints on the potential post-translational regulation of TXNIP have emerged from system proteomic studies. For example, Thr349 (PTpTPL-) or Ser361 (QDpSPIF-) were identified as potential phosphorylation sites in a high-through proteome-wide mapping of protein mitotic phosphorylation sites by mass spectrometry (31), suggesting that TXNIP could be phosphorylated at those two conserved sites by an unknown proline-directed kinase(s).

Importantly, TXNIP has also been found to undergo ubiquitination in vivo in a global ubiquitination analysis in HeLa cells by mass spectrometry. And Lys122 was detected as a one of potential ubiquitin attachment sites (32). However, the ubiquitin E3 ligase responsible for TXNIP ubiquitination has remained unknown.

We searched for the ubiquitin E3 ligase that targets TXNIP for ubiquitination and degradation. TXNIP have two characteristic arrestin domains and a C-terminal domain of unknown function. Structurally, TXNIP belongs to the recently identified alpha arrestin protein family. In the human genome, this protein family has six members: TXNIP and five other proteins, which have been named ARRDC1-5 (Arrestin Domain Containing 1-5). Recently, the function of alpha arrestins (ARTs) in yeast was reported and these proteins were identified as a family of arrestin-related protein that target specific plasma membrane proteins for endocytic down-regulation by serving as adaptors for Rsp5, the Nedd4-like ubiquitin ligase in yeast (33). ART1 contains two PPXY motifs in the C-terminal region, similar to TXNIP. These motifs were essential for recruitment of Rsp5 that ubiquitinates ARTs as well as plasma membrane proteins (33). The presence of two conserved PPXY motifs in the C-terminal region of TXNIP prompted us to investigate whether Rsp5 like E3 ubiquitin ligase(s) in human cells might target TXNIP for ubiquitination and proteasome degradation. Yeast Rsp5 belongs to Nedd4-like ubiquitin ligase family. Yeast has only a single member Rsp5. In humans, this family contains a number of proteins (16). Thus, we tested a panel of human Nedd4-like ubiquitin ligases for their effects on TXNIP stability.

Although the Nedd4-like ubiquitin ligases show similar domain architecture and significant sequence similarity among one another, only Itch was found to promote TXNIP degradation efficiently, underscoring high specificity of Itch for TXNIP. In support of the notion that Itch controls the steady-level of TXNIP through the ubiquitin-proteasome pathway, we also found that (1) Treatment of 293T or U2OS cells with proteasome inhibitors MG132 and lactacystein caused TXNIP protein accumulation, suggesting that TXNIP is normally degraded by the proteasome. (2) TXNIP was shown to form a stable complex with Itch. (3) TXNIP undergoes Itch-dependent ubiquitination by in vivo and in vitro.

Thioredoxin exhibits an anti-apoptotic activity by scavenging ROS, or promoting ubiquitination and degradation of signal regulating kinase-1 (ASK-1). As an inhibitor of thioredoxin, TXNIP promotes apoptosis (1). Indeed, up-regulation of TXNIP expression was associated with the induction of apoptosis triggered by various apoptosis-inducing agents. Moreover, TXNIP overexpression was sufficient to induce apoptosis in WEHI7.2 T cell and primary rat cardiomyocyte (29,34). Interestingly,
ubiquitin E3 ligase Itch has been implicated in apoptosis. Itch null MEF cells are more sensitive to DNA damaging reagents-induced apoptosis compared to wild type MEF (35). The anti-apoptotic function of Itch has been attributed in large part to the ubiquitination and degradation of two key transcription factors, p63 and p73, which are pro-apoptotic (23,36). Our findings raised the possibility that TXNIP may serve as an alternative mediator of the anti-apoptotic function of Itch in addition to p63 and p73.

The identification of Itch as the E3 ligase for ubiquitination and degradation of TXNIP also shed new light on the roles of these proteins in cancer. TXNIP is a novel tumor suppressor and its expression is dramatically reduced in various tumor tissues, including breast, lung, colon, gastrointestinal and prostate cancers. TXNIP null mouse shows higher incidence of hepatocellular carcinoma (7). In contrast, Itch is an amplification target detected in anaplastic thyroid carcinoma cells (37). Queries in Oncomine database also revealed that Itch is up-regulated in lymphoma, bladder and breast cancers. Although it is reported promoter methylation and histone deacetylation may be one possible mechanism in the downregulation of TXNIP in human cancers, it is also possible that an increase in Itch expression can also play a part in TXNIP downregulation.

Itch knockout mice have been generated and Itch null mouse displays epidermis abnormality (18). The epidermis phenotype has been explained by the disregulation of a number of Itch ubiquitination targets that are key transcription factors controlling epidermal stem cell maintenance and keratinocyte differentiation, such as C-Jun, p63, Notch and Gli. Intriguingly, in addition to thioredoxin, TXNIP was also found to interact with Sciellin, a precursor of the cornified envelope, and may play a role in regulating the transition of postmitotic keratinocytes to differentiating ones (38). TXNIP is present in all layers of the epidermis with higher abundance in the upper layers. It will be interesting to investigate if Itch controls the in vivo expression gradient of the TXNIP in epidermis layers and if this control would have a role in epidermal keratinocyte differentiation.

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We thank Dr. Junji Yodoi for flag-TXNIP construct, Dr. Gerry Melino for Myc-Itch and Myc-Itch /C830A constructs, Annie Angers for GFP-Itch and GST-Itch construct, Kohei Miyazono Myc-Smurfl/2 and Myc-WWP1 constructs, and Xuejun Jiang for HA-nedd4 construct.
REFERENCES


FOOTNOTES

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The abbreviations used are: TXNIP, Thioredoxin interacting protein; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; PBS, phosphate-buffered saline; GST, glutathione S-transferase; Ni-NTA, nickel-nitrilotriacetic acid; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; CHX, cycloheximide; ROS, Reactive oxygen species.

FIGURE LEGENDS

FIGURE 1. TXNIP stability is controlled by the ubiquitin-proteasome pathway. (A) U2OS cells and 293T cells were treated with 20 μM MG132 (upper panel and middle panel), or with 10 μM Lactacystin (lower panel, U2OS cells only) for indicated lengths of times. Equal amounts of total cell lysates were subjected to Western blot using antibodies against TXNIP and Actin, respectively. The mean value (± SD) of three independent experiments is shown.
(B) qRT-PCR measurements of the mRNA levels of TXNIP in U2OS cells after treatment with lactacystin at the indicated time points. (C) left panel: HA-TXNIP and (His)$_6$-Ub constructs were transfected into H1299 cells. (His)$_6$–ubiquitinated proteins were purified and subjected to Western blot using anti-TXNIP antibody. The lower panel contains control cell lysates. right panel: HA-TXNIP was transfected into H1299 cells. left panel: Flag-TXNIP expression plasmid was transfected into H1299 cells. TXNIP proteins were immunoprecipitated by Flag-M2 antibody after treatment with 40 μM MG132 for 4 h. The immunoprecipitates were eluted using 3X Flag peptide and were separated by SDS-PAGE. The polyubiquitinated forms of TXNIP were detected by Western blot with anti-TXNIP antibody. (D) Flag-TXNIP was co-transfected into H1299 cells with wild type Ub or ubiquitin mutant (K48R, K48 only, K63R, K63 only, and KO). TXNIP proteins were immunoprecipitated by Flag-M2 antibody. The immunoprecipitates were eluted using 3X Flag peptide and were separated on SDS-PAGE. The polyubiquitinated forms of TXNIP were detected by Western blot with anti-Ub antibody. The pGFP–N3 expression construct was included as a control.

FIGURE 2. Itch promotes TXNIP degradation. (A) Sequence alignment of TXNIP orthologs from different species, including Homo sapiens, Mus musculus, Canis familiaris, Xenopus tropicalis, Tetraodon nigroviridis. The two conserved PPXY motifs were boxed. (B) Expression construct for HA-TXNIP was co-transfected with increasing amounts of Myc-Itch, Myc-Smurf1, Myc-Smurf2, Myc-WWP1, and Myc-Nedd4 expression constructs into H1299 cells. The protein levels of TXNIP and E3 ubiquitin ligases were determined by Western blot with anti-TXNIP and anti-Myc antibodies, respectively. The pGFP–N3 expression construct was included as a transfection efficiency control and levels of GFP were determined by Western blot with anti-GFP antibodies. The quantification of immunoblot is shown in the lower panel. The mean value (± SD) of three independent experiments is shown.

FIGURE 3. Itch regulates TXNIP stability. (A) The same amount of HA-TXNIP expression construct was co-transfected with increasing amounts of Myc-Itch, Myc-Itch/C830A mutant into H1299 cells. The protein levels of TXNIP and Itch were determined by Western blot with anti-TXNIP and anti-Itch antibodies, respectively. The pGFP–N3 expression construct was included as a transfection efficiency control and levels of GFP were assessed with GFP antibodies. (B) HA-TXNIP was transfected either alone or in combination with Myc-Itch, Itch C830A expression plasmids into H1299 cells. After a 24 h incubation, cells were treated with 40 μM of the proteasome inhibitor MG132 or DMSO for 6 h. The protein levels of TXNIP and Itch were determined by Western blot with anti-TXNIP and anti-Itch antibodies, respectively. The pGFP–N3 expression construct was included as a control similar to (A). (C) H1299 cells were co-transfected with HA-TXNIP, along with empty vector or Itch, Itch/C830A mutant. Twenty four hours after transfection, cells were treated with 30 μM CHX for indicated lengths of time. Equal amounts of cell lysates were subjected to Western blot with anti-TXNIP and anti-Actin antibodies, respectively. (D) For each experimental condition, the blots correspond to one representative experiment and the graph shows the quantification of TXNIP levels using Actin for standardization. The error bars represent mean values ± SD (error bar) from three independent experiments. (E) 293T cells were transfected with
Myc-Itch or Itch/C830A expression constructs. Thirty six hours after transfection, cells were harvested and lysed. The cell lysates were subjected to Western blot with anti-TXNIP and anti-Itch antibodies, respectively. (F) 293T cells and U2OS cells were transiently transfected with two Itch-specific siRNA or control siRNA. Forty-eight hours after transfection, the protein levels of endogenous TXNIP and Itch were determined by Western blot with anti-TXNIP and anti-Itch antibodies, respectively. The quantification of immunoblots are shown in the lower panel. The mean values (± SD) of three independent experiments are shown. (F) qRT-PCR measurements of the mRNA level of Itch and TXNIP in Itch RNAi U2OS cells. Two Itch-specific siRNA or control siRNA was transfected into U2OS cells. The left three columns are relative mRNA levels of TXNIP, and the right three columns correspond to those of Itch. The mRNA level of GAPDH was used for normalization. All data shown are mean values ± SD (error bar) from three independent experiments.

FIGURE 4. TXNIP forms a complex with Itch. (A, B) H1299 cells were transiently co-transfected with HA-TXNIP and Myc-Itch or Itch/C830A constructs. Cell lysates were prepared and subjected to immunoprecipitation with TXNIP (A) or Myc (B) antibodies, respectively. The immunoprecipitates were analyzed by Western blot with anti-TXNIP and anti-Myc antibodies, respectively. (C) Endogenous TXNIP interacts with endogenous Itch in 293T Cells. 293T cell lysates were incubated with Protein A/G Sepharose conjugated with either control IgG or TXNIP antibody. The immunoprecipitates were washed and bound proteins were resolved on SDS-PAGE followed by Western blot with TXNIP and Itch antibodies, respectively. (D) Colocalization of TXNIP and Itch in COS-7 cells. COS-7 cells were transiently transfected with HA-TXNIP and GFP-Itch constructs either alone or in combination. TXNIP was stained in paraformaldehyde-fixed cells with mouse anti-HA antibodies followed by incubation with Texas Red-conjugated goat anti-mouse second antibodies. Nuclei were stained with DAPI.

FIGURE 5. Itch and TXNIP interacts through WW domains and PPXY motifs. (A, B) Deletion constructs of TXNIP (A) and Itch (B) are shown schematically. (C) The second PPXY motif in TXNIP is required for its binding to Itch. Bacterially expressed GST fusion proteins of wild type (WT), single (∆PY1 or ∆PY2), double (∆PY1/2) and single point (PYF) mutants of TXNIP were bound to glutathione-Sepharose beads as indicated and incubated with lysates of H1299 cells transfected with a Myc-Itch expression plasmid. Bound Myc-Itch, GST-TXNIP (bottom) were subjected to Western blot with anti-Itch and anti-GST antibodies, respectively. (D) Bacterially expressed GST fusion proteins of wild type (WT), C2 domain (C2), WW domain (WW), HECT domain (HECT) mutants of TXNIP were bound to glutathione-Sepharose beads and incubated with lysates of 293T cells transfected with the HA-TXNIP expression construct. Bound TXNIP, GST-Itch were detected by Western blot with anti-TXNIP and anti-GST antibodies, respectively. (E) The same amount of WT or various mutant expression constructs of TXNIP were co-transfected with increasing amounts of Itch plasmid into H1299 cells. The protein levels of TXNIP and Itch were determined by Western blot with TXNIP and Itch antibodies, respectively. The pGFP–N3 expression construct was included as a transfection efficiency control, and levels of GFP were
determined by Western blot with anti-GFP antibodies. The quantification of immunoblot is shown in the lower panel. The mean value (± SD) of three independent experiments is shown.

FIGURE 6. Itch ubiquitnates TXNIP both in vivo and in vitro. (A) Itch ubiquitnates TXNIP in vivo. Flag-TXNIP, HA-Ubiquitin and Myc-Itch, or Itch/C830A mutant constructs were co-transfected into H1299 cells. TXNIP proteins were immunoprecipitated by Flag-M2 antibody. The immunoprecipitates were eluted using 3X Flag peptide and were resolved by SDS-PAGE. The polyubiquitinated forms of TXNIP were detected by Western blot with anti-Ub antbody. The pGFP-N3 expression construct was included as a control. (B) Itch directly ubiquitinates TXNIP in vitro. Bacterially expressed and purified GST-TXNIP were incubated with GST-Itch or GST-Itch/C830A mutant in the presence of E1, E2 (UbcH7) and ubiquitin (His-Ub). Following the ubiquitination reaction, the TXNIP-ubiquitin conjugates were detected by Western blot with anti-TXNIP antbody.

FIGURE 7. Itch modulates intracellular ROS by controlling TXNIP protein levels. (A) U2OS cells were transiently transfected with two Itch-specific siRNA and one TXNIP-speicific siRNA either alone or in combination. Forty eight hours after transfection, cells were trypsinized and H2-DCF-DA was then added for an additional 30 min. Labeled cells were analyzed by flow cytometry. The mean value (± SD) of three independent experiments is shown. * indicates statistical significance (*:P < 0.05) (B) The protein levels of endogenous TXNIP and Itch were determined by Western blot with anti-TXNIP and anti-Itch antibodies, respectively.

FIGURE 8. Knockdown of Itch promotes etoposide-induced ROS elevation and apoptosis. (A) U2OS cells were transiently transfected with two Itch-specific siRNA or one TXNIP-speicific siRNA. Forty eight hours after transfection, cells were treated with DMSO or 20 µM Etoposide for 12 h. Cells were trypsinized, H2-DCF-DA was then added for an additional 30 min. Labeled cells were analyzed by flow cytometry. (B) Similar to (A), U2OS cells were treated with DMSO or 20 µM etoposide for 36 h. Cells were stained with PI, followed by FACS analysis. The mean value (± SD) of three independent experiments is shown. * indicates statistical significance (*:P < 0.05)
Fig. 6

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WB: α-TXNIP

TXNIP
Fig. 7

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50 -

α-Itch

α-TXNIP

α-Actin
Fig. 8

A

![Graph A showing ROS levels with different conditions](image)

B

![Graph B showing Sub-G1 levels with different conditions](image)
The ubiquitin ligase itch regulates apoptosis by targeting thioredoxin-interacting protein for ubiquitin-dependent degradation
Pingzhao Zhang, Chenji Wang, Kun Gao, Dejie Wang, Jun Mao, Jian An, Chen Xu, Di Wu, Hongxiu Yu, Jun O Liu and Long Yu

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