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Ubiquitin ligase ITCH recruitment suppresses the aggregation and cellular toxicity of cytoplasmic misfolded proteins

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The protein quality control (QC) system protects cells against cellular toxicity induced by misfolded proteins and maintains overall cellular fitness. Inefficient clearance of or failure to degrade damaged proteins causes several diseases, especially age-linked neurodegenerative disorders. Attenuation of misfolded protein degradation under severe stress conditions leads to the rapid over-accumulation of toxic proteinaceous aggregates in the cytoplasmic compartment. However, the precise cytoplasmic quality control degradation mechanism is unknown. In the present study, we demonstrate that the Nedd4-like E3 ubiquitin ligase ITCH specifically interacts with mutant *bona fide* misfolded proteins and colocalizes with their perinuclear aggregates. In a cell culture model, we demonstrate ITCH recruitment by cytoplasmic inclusions containing polyglutamine-expanded huntingtin or ataxin-3 proteins. Transient overexpression of ITCH dramatically induced the degradation of thermally denatured misfolded luciferase protein. Partial depletion of ITCH increased the rate of aggregate formation and cell death generated by expanded polyglutamine proteins. Finally, we demonstrate that overexpression of ITCH alleviates the cytotoxic potential of expanded polyglutamine proteins and reduces aggregation. These observations indicate that ITCH is involved in the cytosolic quality control pathway and may help to explain how abnormal proteins are targeted by QC ubiquitin-protein ligases.

A human cell retains billion protein molecules that face continuous risk of misfolding. Aggregation of misfolded proteins causes multifactorial toxicity and defects. Accumulation of damaged proteins is the primary clinical hallmark of several neurodegenerative diseases^{1,2}. The protein QC system cleans the cell and efficiently maintains proteostasis with the help of chaperones, the ubiquitin proteasome system (UPS) and the autophagy pathway^{3,4}. The protein QC system functions to retain the competence of the first to last lines of proteolytic defense against the deleterious aggregation of abnormal proteins in various cellular compartments. Therefore, identification of key molecular steps that facilitate misfolded protein recognition or crucial substrate selectivity and induce their destruction, will help in the design of efficient cellular strategies to target the protein QC system. Molecular chaperones promote the folding of nascent polypeptides, the UPS enhances the selective elimination of misfolded or old proteins, and autophagy clears the accumulation of long-lived, defective proteins⁵⁻⁷.

Damaged or poor proteins overall decrease fitness of cells. However, so far, no widely used cytoplasmic protein QC mechanism has been clearly described and how the cellular QC system achieves the selective elimination of misfolded proteins remains unclear. Cytoplasmic misfolded protein clearance is crucial for cytoplasm-native proteins and aberrant secretory proteins targeted in the ER⁸⁻¹⁰. Recently, it has been shown that the UPS and selective autophagy govern the degradation of many abnormal proteins in a selective manner with the help of a few E3 ubiquitin ligases^{11,12}. Ubiquitination of a target protein is a multi-step process and begins with the help of ATP when the ubiquitin-activating enzyme E1 adenylates ubiquitin (76-aa) on its C terminus at the glycine residue. Then, activated ubiquitin is transferred to ubiquitin-conjugating enzymes known as E2s. Finally, E2-bound ubiquitin is transferred to an ϵ -amino group of a lysine residue in a crucial substrate protein, or repeated ubiquitylation produces a polyubiquitin chain via E3 ubiquitin ligase activity¹³. In this entire cascade, E3 ubiquitin ligase provides specific substrate selectivity in the ubiquitination process, and more than 500 E3 ubiquitin ligases have been identified in mammalian cells¹⁴⁻¹⁶. This strongly suggests that possibly a hub of E3 ubiquitin ligase generate checkpoints for the aggregation of aberrant proteins.

Recently, we have shown that Mahogunin RING finger protein 1 (MGRN1) promotes the degradation of abnormal proteins with the help of Hsp70 chaperones and also suppress expanded polyglutamine proteins



mediated toxicity^{17,18}. Carboxy Terminus of Hsp70-Interacting Protein (CHIP) E3 ubiquitin ligase targets polyglutamine expansion proteins for degradation and reduces aggregation in a zebrafish model of polyglutamine disease^{19,20}. ER-associated E3 ubiquitin ligase Autocrine Motility Factor Receptor (AMFR) or Gp78 induces the degradation of Superoxide Dismutase 1 (SOD1) and ataxin-3 and provides cytoprotection against mutant SOD1-induced toxicity²¹. E6-Associated Protein (E6-AP) is another E3 ubiquitin ligase that is recruited by aggregates and also stimulates the degradation of mutant SOD1, α -synuclein and expanded polyglutamine proteins^{22–25}. Parkin E3 ubiquitin ligase, which is often mutated in Parkinson's disease, interacts with expanded polyglutamine proteins and promotes their degradation²⁶. Leucine-Rich Repeat And Sterile Alpha Motif-Containing Protein 1 (LRSAM1) is an LRR and RING domain E3 ubiquitin ligase that recognizes intracellular *Salmonella typhimurium* during ubiquitin-mediated autophagy and protects the cytoplasm against pathogens²⁷. LISTERIN is another RING finger domain E3 ubiquitin ligase, and the *lister* gene is crucial for embryonic development; its perturbation in mice causes progressive neurodegeneration²⁸. Numerous E3 ubiquitin ligases have been identified in the cellular QC pathway, and Hsp70 assists San1p ubiquitin ligase to degrade cytoplasmic misfolded proteins²⁹.

Currently, it is known that the cytoplasm contains a dense pool of old or damaged proteins, but the cytoplasmic QC mechanism is not very clear. The precise mechanism by which E3 ubiquitin ligases specifically recognize misfolded proteins in the cytoplasm and how they promote their degradation is still not known. Here, for the first time, we observed that Itchy E3 Ubiquitin Protein Ligase (ITCH), a member of the Nedd4 family of HECT domain E3 ubiquitin ligases, recruits both model misfolded proteins and expanded polyglutamine proteins in the cytoplasmic compartment. Our current study explored for the first time the QC function of ITCH with respect to cytoplasmic misfolded proteins. Here, we demonstrate that ITCH specifically interacts with model misfolded proteins and induces the degradation of heat-denatured luciferase proteins. Over all previous findings and current study suggest that cells have a powerful proteome and QC system to hinder the aggregation of misfolded proteins, and a better understanding of QC E3 ubiquitin ligases may allow the protective response under proteotoxic stress conditions to be enhanced.

Results

Cellular stress-induced misfolding triggers ITCH upregulation and recruitment with cytosolic-misfolded protein inclusions.

The aggregation of misfolded proteins has been implicated in the etiology of aging, neurodegenerative diseases and other protein conformational disorders³⁰. To overcome this multifactorial problem, the cellular protein QC system facilitates protein folding and induces the elimination of abnormal proteins. A few E3 ubiquitin ligases play a pivotal role in this QC pathway, but no detailed QC pathway has been described for the clearance of accumulated aberrant proteins from the dense cellular pool. To elucidate the molecular function of E3 ubiquitin ligases in the QC pathway, we treated cells with various stress-inducing agents. Interestingly, endogenous levels of ITCH E3 ubiquitin ligase were increased after autophagy dysfunction (chloroquine and bafilomycin) and after HS (heat stress) exposure (Fig. 1A). We noticed an approximately 0.5-fold increase in ITCH levels under various stress conditions compared to the control (Fig. 1B). This preliminary result indicates that stress-induced protein misfolding causes an increase in the endogenous level of ITCH E3 ubiquitin ligase mediated by the QC pathway. To investigate this possibility, we performed an immunofluorescence analysis under proteasomal dysfunction and autophagy inhibitory conditions. We observed numerous small (Baf) and robust large (MG132 and CQ)

cytoplasmic inclusions of the ITCH protein near the periphery of the nuclear region (Fig. 1C).

We further characterized ITCH recruitment using misfolded proteins in cells. As previously described³¹, we used green fluorescent protein (GFP) fusions of wild-type chloramphenicol-acetyltransferase (wtCAT) and mutant GFP- Δ 9CAT proteins in several experiments. GFP- Δ 9CAT forms perinuclear aggregates and structures similar to cytoplasmic inclusion bodies; however, GFP-wtCAT is distributed diffusely in cells. Using fluorescence microscopy analysis, we observed that induced ITCH colocalized with GFP- Δ 9CAT perinuclear inclusions, and these inclusions were also positive for ubiquitin staining (Fig. 1D and 1E). Both nascent and pre-existing polypeptides bear a constant risk of misfolding and aggregation, and these aberrant proteins are cleared by the UPS or by autophagy^{32–34}. To further characterize these results, we performed immunofluorescence analysis of cells expressing GFP-wtCAT and GFP- Δ 9CAT proteins with p62 (early autophagic structures) and 20S (proteasome marker). We observed a marked increase in the levels of ITCH protein and the recruitment and specific colocalization of ITCH with p62- (Fig. 1F) and 20S- (Fig. 1G) positive cytoplasmic GFP- Δ 9CAT misfolded protein aggregates. Overall, these results demonstrate the possible involvement of ITCH in the cellular QC pathway and recited into cytosolic aberrant aggregates.

ITCH interacts with aberrant or misfolded proteins. Ubiquitination is a well-known process of the cellular QC pathway³⁵. We examined the localization of ITCH with cytoplasmic misfolded proteins, and we hypothesized that ITCH could be associated with misfolded proteins. To test this hypothesis, we first assessed the interaction between ITCH and misfolded proteins. Therefore, we expressed GFP-wtCAT and GFP- Δ 9CAT constructs in cells, prepared cell lysates 48 h later, and performed a detailed immunoprecipitation assay. As shown in Fig. (2A), GFP- Δ 9CAT-expressing cells were positive for cytoplasmic aggregates, and immunoblot analysis determined the ubiquitination profile of the misfolded (GFP- Δ 9CAT) proteins (Fig. 2B). The same cell lysates were pulled down by an anti-ITCH antibody, and the blots were sequentially developed using anti-GFP and anti-ITCH antibodies. This result demonstrates the specific interaction of ITCH with GFP- Δ 9CAT misfolded proteins (Fig. 2C). Next, we further characterized the interaction between ITCH and GFP- Δ 9CAT misfolded proteins in a reverse immunoprecipitation experiment. We pulled down the same cell lysates using an anti-GFP antibody, and the blots were developed using anti-ITCH and anti-GFP antibodies (Fig. 2D). To validate this novel interaction, we performed a detailed immunoprecipitation study with various controls. GFP-wtCAT and GFP- Δ 9CAT proteins were pulled down using beads only (control) and normal IgG, and the blots were incubated with anti-GFP (Fig. 2E) and anti-ITCH antibodies (Fig. 2F). Together with the findings of the experiments described above, these results indicate that ITCH interacts with misfolded proteins. These results also support our current immunofluorescence observation of ITCH-specific recruitment by cytosolic misfolded inclusions of GFP- Δ 9CAT proteins.

ITCH colocalizes with cytoplasmic polyglutamine-containing huntingtin aggregates.

Ubiquitin is a pivotal component of misfolded inclusion bodies, which suggests that ubiquitin has a crucial role in the elimination of misfolded proteins via the QC pathway. In this study, we postulated that ITCH may colocalize with and recruit misfolded inclusions of expanded huntingtin polyglutamine aggregates. To determine whether ITCH colocalizes with huntingtin-expanded polyglutamine aggregates in cells, we performed immunofluorescence staining of EGFP-HDQ74-expressing cells using the anti-ITCH antibody, as shown in Fig. 3A–3B, and we found that ITCH is recruited more by cytoplasmic inclusions of huntingtin-expanded polyglutamine aggregates than by the protein with the normal amount of glutamine repeats (EGFP-HDQ23).

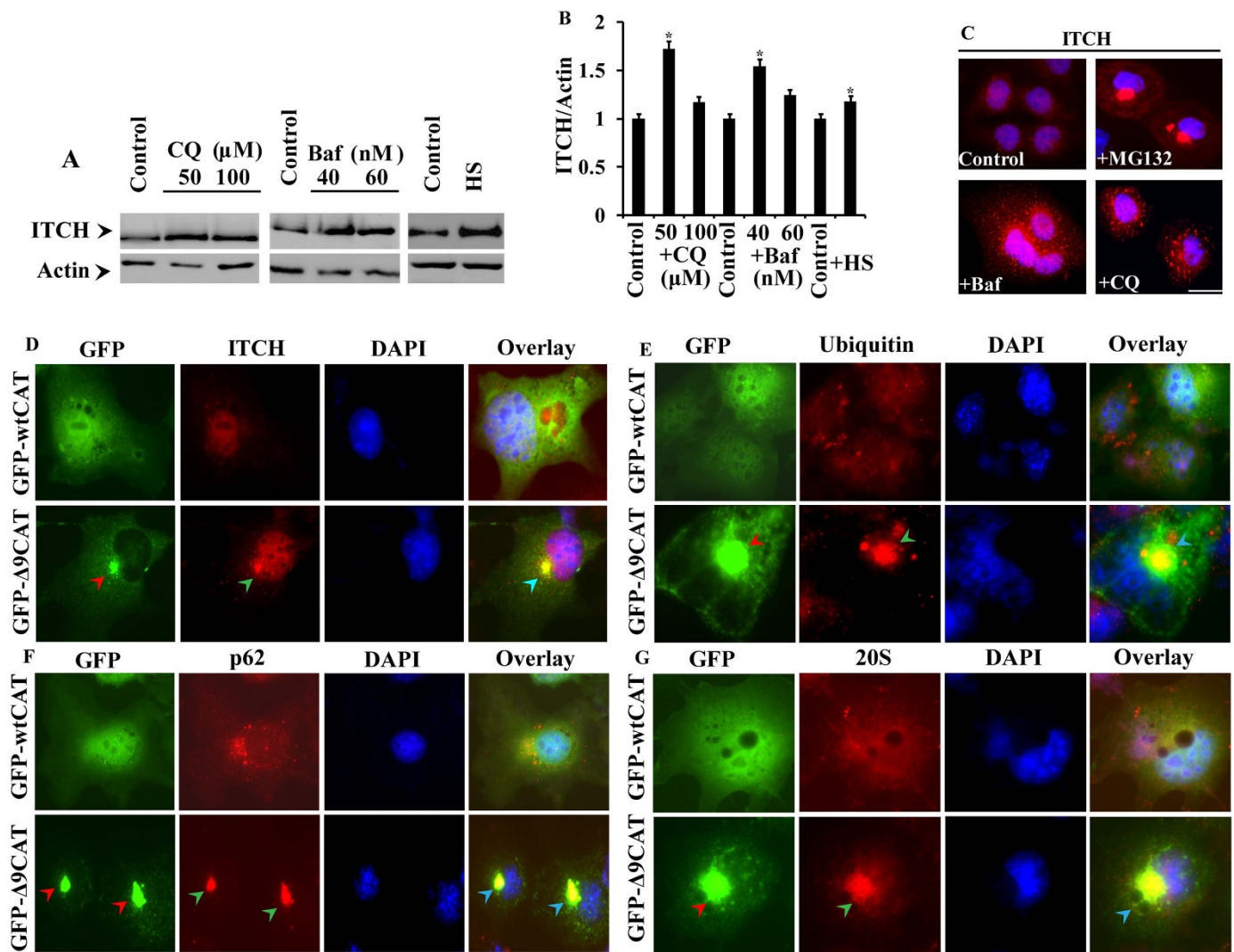


Figure 1 | ITCH is induced under various stress conditions and redistributes with perinuclear cytoplasmic misfolded aggregates. (A) Cos-7 cells were plated into six-well culture plates and treated with different doses of chloroquine (CQ) for 8 h and bafilomycin (Baf) for 12 h, as shown in section A. Effect of heat stress (HS) on ITCH levels. Cells were exposed to 43°C for 30 minutes and then returned to normal incubation conditions for 3 h. The cell lysates were then collected and processed for immunoblotting using anti-ITCH and anti-actin antibodies. (B) Quantification of ITCH band intensities collected from three different experiments as shown in A using NIH image analysis software. *, $P < 0.05$ compared with the untreated control. (C) Cells were plated into two-well chamber slides, and on the subsequent day, they were treated with 10 μM MG132 for 8 h, 50 nM Baf for 10 h and 40 μM CQ for 5 h. (D–G) The Cos-7 cells were transiently transfected with the indicated (GFP-wtCAT and GFP-Δ9CAT) plasmids. After 48 h of transfection, the cells were subjected to immunofluorescence staining using antibodies against ITCH (D), ubiquitin (E), p62 (F) and 20S proteasome (G). The arrows indicate the colocalization of ITCH with perinuclear cytoplasmic aggregates of GFP-Δ9CAT misfolded proteins. A rhodamine-conjugated secondary antibody was used to stain ITCH (D), Ub (E), p62 (F) and 20S (G). Full-length blots are presented in Supplementary Figure-S1. Scale bar, 20 μm.

Furthermore, we observed that cytosolic aggregates of misfolded EGFP-HDQ74 are also positive for ubiquitin staining (Fig. 3C–3D). Several components of protein QC pathways and transcription factors have been found to be sequestered with misfolded proteins and with expanded polyglutamine repeats proteins in cellular models^{36,37}. We determined whether polyubiquitinated ITCH-positive cytosolic inclusions of expanded huntingtin polyglutamine aggregates retain the capability to activate components of autophagy and the UPS pathway. In particular, we used sets of cells similar to those described above for the immunofluorescence analysis of LC3 (autophagosome marker), p62 (early autophagic structures) and 20S (proteasome marker). As shown in Fig. 3E–3J, all cytoplasmic EGFP-HDQ74 misfolded protein aggregates were positive for LC3 (Fig. 3F), p62 (Fig. 3H) and 20S (Fig. 3J), in contrast to their respective controls (EGFP-HDQ23 with normal

glutamine repeats). These results are consistent with our findings that ITCH specifically recruits cytosolic misfolded proteins.

Colocalization of ITCH with cytoplasmic aggregates of ataxin-3-expanded polyglutamine protein. At least nine neurodegenerative diseases are caused by polyglutamine-containing expansions^{38,39}. In this study, we showed that the ITCH E3 ubiquitin ligase interacts with misfolded proteins and colocalizes with huntingtin-expanded polyglutamine proteins. To assess whether another externally applied polyglutamine expansion protein colocalizes with ITCH, we used normal ataxin-3(28Q) and expanded-polyglutamine ataxin-3(84Q) proteins for immunofluorescence staining. Upon expression in cells, cytosolic large inclusions of polyglutamine-expanded ataxin-3(84Q) colocalized with ITCH and were positive for ubiquitin staining (Fig. 4A–4D). In various species, distinct

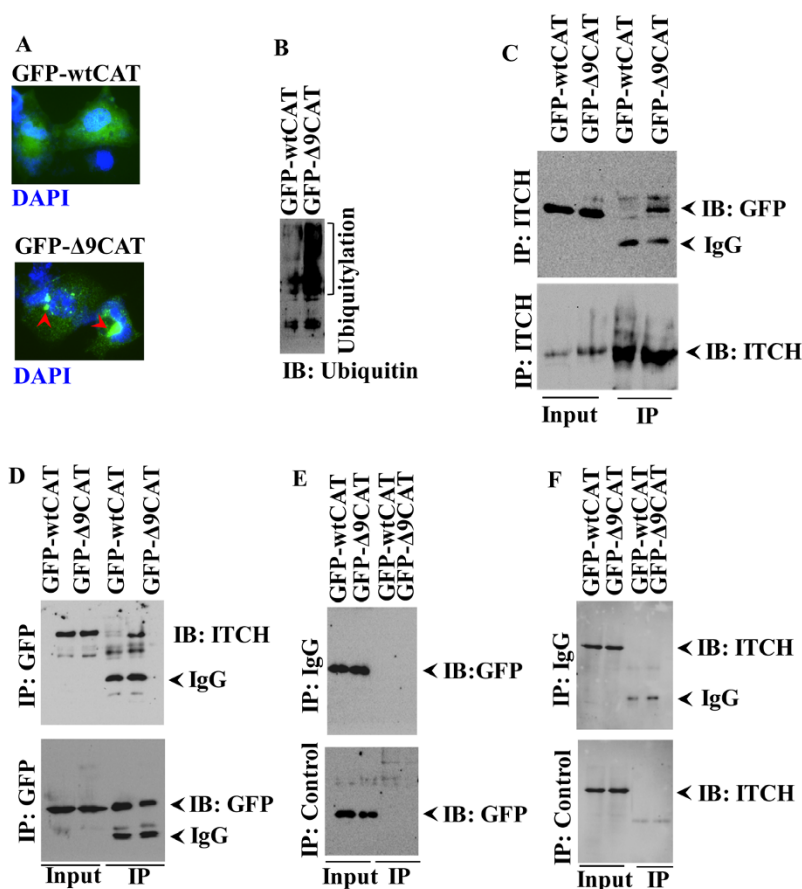


Figure 2 | Interaction of ITCH with misfolded proteins. (A–B) Transient transfection was used to overexpress GFP-wtCAT and GFP- Δ 9CAT constructs, and the cells were processed for immunofluorescence staining with nuclear staining using DAPI (A). The same sets of cell lysates were probed with an anti-ubiquitin antibody (B). (C) GFP-wtCAT- and GFP- Δ 9CAT-transfected cell lysates were pulled down by an anti-ITCH antibody, and the blots were developed using an anti-GFP antibody (upper panel) and anti-ITCH antibody (lower panel). (D) The same transfected samples were processed for immunoprecipitation with the anti-GFP antibody, and the blots were processed for immunoblotting using anti-ITCH (upper panel) and anti-GFP antibodies (lower panel). (E–F) The cells were transfected as described in A and 48 h after transfection, cell lysates were prepared and processed for immunoprecipitation (IP) using beads only (control); immunoprecipitates were then probed with anti-GFP (E) and anti-ITCH antibodies (F).

cytotoxic cellular aggregates are formed in an organized manner with specific characteristics (e.g., colocalization with UPS components, microtubules, transcription factors including TATA binding protein (TBP) and CREB binding protein (CBP) and autophagy components)^{40–44}. Therefore, we next examined the possibility of interactions between ITCH-positive expanded-polyglutamine ataxin-3 proteins and other components of the cellular QC pathway. As shown in Fig. 4E–J, cytosolic inclusion bodies of expanded-polyglutamine ataxin-3 proteins exhibited strong immunoreactivity with LC3 (Fig. 4F), p62 (Fig. 4H) and 20S (Fig. 4I), whereas ataxin-3(28Q) was soluble and diffused throughout both compartments. This analysis provides new insight that ITCH E3 ubiquitin ligase generally recruits the cytoplasmic misfolded inclusions. Sequestration of ITCH by aggregates most likely results in a loss of function of this normal protein and generates multifactorial cellular toxicity.

ITCH interacts with expanded polyglutamine proteins. Under cytotoxic conditions, several E3 ubiquitin ligases recognize abnormal or misfolded proteins via molecular interactions to promote the clearance of such proteins, which helps maintain the correct proteostasis in cells^{45,46}. Here, we observed that ITCH colocalizes with cytoplasmic misfolded inclusions, and this recruitment may represent an attempt to cope with misfolded proteins that are not refolded or aggregated and to sequester these proteins into QC compartments. Next, we investigated the interaction between ITCH and misfolded expanded-polyglutamine proteins. As shown in Fig. 5A,

we used cells expressing aggregates of proteins for a coimmunoprecipitation assay. We incubated the anti-GFP antibody with cell lysates obtained from expanded-polyglutamine ataxin-3-expressing cells for the coimmunoprecipitation assay, and the blots were sequentially probed with anti-GFP and anti-ubiquitin antibodies (Fig. 5B). To confirm this novel interaction, we used another polyglutamine protein (i.e., expanded-polyglutamine huntingtin), and the lysates from the same cells were pulled down by the anti-ITCH antibody. The blots were developed using anti-GFP and anti-ITCH antibodies. We observed strong and specific interaction of ITCH with insoluble SDS higher-molecular-weight species of expanded-polyglutamine proteins but not with proteins containing normal repeats (Fig. 5C). Furthermore, to investigate the misfolding of expanded-polyglutamine proteins, we pulled down ataxin-3 polyglutamine proteins using the anti-ITCH antibody, and the blots were sequentially developed with anti-GFP and anti-ITCH antibodies. We observed only the interaction of ITCH with expanded-polyglutamine ataxin-3(84Q) proteins (Fig. 5D). To examine the interaction between ITCH and expanded-polyglutamine proteins, we performed detailed control experiments using beads only (control), and the blots were probed with the anti-GFP antibody (Fig. 5E–5F). These results suggest that ITCH most likely interacts with expanded-polyglutamine proteins.

ITCH overexpression suppresses aggresome formation and alleviates proteotoxicity induced by expanded polyglutamine proteins.

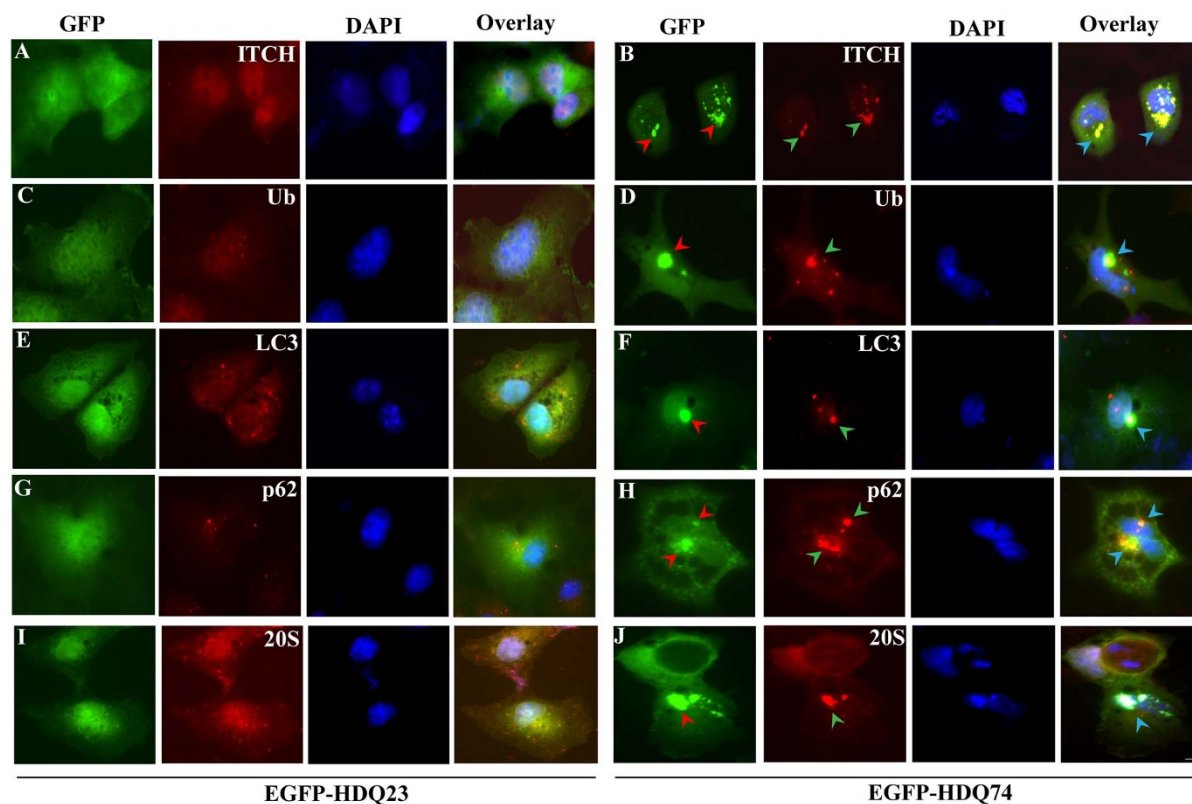


Figure 3 | Recruitment of ITCH with cytoplasmic expanded-polyglutamine huntingtin inclusion bodies. (A–B) Cos-7 cells were plated onto two-well chambers and transiently transfected with EGFP-HDQ23 (A) and EGFP-HDQ74 (B) constructs. Forty-eight hours after transfection, the cells were subjected to immunofluorescence staining with the anti-ITCH antibody. (C–D) Colocalization of ubiquitin (red) with cytoplasmic inclusions of misfolded polyglutamine EGFP-HDQ74 (D) proteins compared to the EGFP-HDQ23 (C) with normal repeats is shown. (E–F) Similar sets of cells were subjected to LC3 (red) staining for normal EGFP-HDQ23 (E) and EGFP-HDQ74 (F) with expanded repeats. (G–H) In several experiments, the cells were processed for immunolocalization of cytoplasmic huntingtin aggregates with p62 (red) in normal (G) and expanded- (H) polyglutamine-expressing cells. (I–J) The cells that expressed EGFP-HDQ23 (I) and EGFP-HDQ74 (J) constructs were subjected to immunofluorescence staining with an antibody targeting the 20S proteasome. A rhodamine-conjugated secondary antibody (red) was used to label ITCH, ubiquitin, LC3, p62 and 20S proteins. The arrows indicate the redistribution of ITCH with cytoplasmic polyglutamine-expanded huntingtin aggregates positive for p62, ubiquitin, LC3 and 20S. The overlay images also include 4',6-diamidino-2-phenylindole (DAPI) staining of the cell nuclei. Scale bar, 20 μ m.

Our current observations demonstrate that ITCH, an E3 ubiquitin ligase, is recruited to cytoplasmic inclusion bodies formed by expanded-polyglutamine proteins and other misfolded proteins. ITCH localization was site-specific in cells, and the reason why ITCH recruits and interacts with cytoplasmic misfolded aggregates is not known. We confirmed the colocalization of inactive mutant ITCH with misfolded and expanded-polyglutamine proteins. In particular, we coexpressed an inactive ITCH mutant (Itch-C822S) with misfolded (GFP-wtCAT and GFP- Δ 9CAT) and expanded-polyglutamine (huntingtin and ataxin-3) constructs to assess their colocalization. To our surprise, the inactive ITCH mutant colocalized with cytoplasmic inclusions of GFP- Δ 9CAT-misfolded protein (Fig. 6A–6B) and expanded polyglutamine inclusions (Fig. 6C–6F). Therefore, most likely ITCH recruitment by cytoplasmic misfolded aggregates is independent of its ubiquitin ligase function.

We further evaluated the functional significance of the interaction of ITCH with misfolded proteins. ITCH is a putative E3 ubiquitin ligase; therefore, we first analyzed its role in the elimination of misfolded proteins. Overexpression of ITCH significantly decreased the levels of both huntingtin (Fig. 7A) and ataxin-3 (Fig. 7B) expanded-polyglutamine proteins, and this effect was inhibited by substitution with the inactive ITCH mutant. A similar set of experiments using a proteasomal inhibitor (MG132) and an autophagy inhibitor (bafilomycin) suggested that ITCH preferentially targets misfolded proteins for proteasomal degradation (Fig. 7C). To further validate the role of

ITCH in the degradation of misfolded proteins, we coexpressed ITCH and its mutant form with a luciferase plasmid. Transfected cells were treated with a 43°C heat shock for 30 min and then returned to 37°C for 2 h of recovery. Experiments were also performed with MG132 and bafilomycin before the heat shock. As demonstrated in Fig. 7D, overexpression of ITCH promotes the degradation of thermally denatured luciferase protein, and this effect was inhibited by treatment with MG132 and bafilomycin. Efficient degradation of heat-denatured luciferase was not observed with mutant ITCH (Fig. 7E). The same sets of samples were used for immunoblot analysis using anti-luciferase and anti-actin antibodies (Fig. 7F).

We next addressed the effects of ITCH overexpression on the number of huntingtin-expanded polyglutamine protein aggregates. As shown in Fig. 7G, overexpression of ITCH significantly decreased the number of huntingtin-expanded polyglutamine protein aggregates in a concentration-dependent manner. To confirm this effect, we knocked down endogenous levels of ITCH using ITCH-specific siRNA in various concentrations, and cells were transfected with a plasmid expressing mutant ITCH. Depletion of ITCH dramatically increased the number of aggregates in cells at different time intervals in a concentration-dependent manner (Fig. 7H). Overexpression and knockdown of ITCH were confirmed by immunoblot analysis using anti-myc and anti-ITCH, respectively (Fig. 7I).

As previously reported, accumulation of misfolded protein inclusions and aggregation of expanded polyglutamine proteins induce multifactorial proteotoxic events in cells and cause cell death^{47–49}.

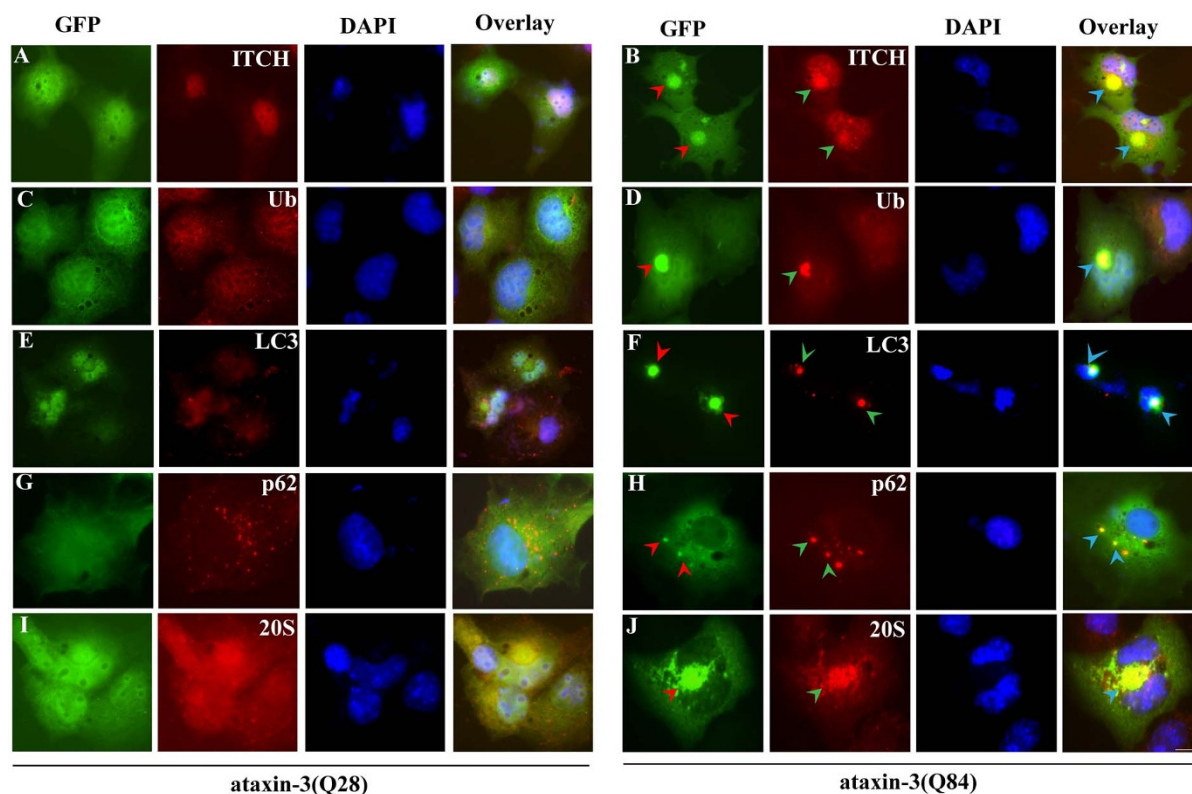


Figure 4 | Recruitment of ITCH by cytoplasmic ataxin-3 aggregates. (A–B) Cells were transiently transfected with ataxin-3-GFP fusion with normal (A) (ataxin-3(Q28)) or expanded (B) polyglutamine repeats (ataxin-3(Q84)). Forty-eight hours after transfection, the cells were processed for immunofluorescence staining with an anti-ITCH antibody (red). (C–D) Inclusion bodies (IBs) containing ataxin-3 with expanded polyglutamine repeats were immunopositive for ubiquitin. Micrographs of Cos-7 cells were transiently transfected with normal (C) ataxin-3(Q28) and expanded- (D) polyglutamine ataxin-3(Q84) plasmids, and the cells were probed with an anti-ubiquitin antibody (red). (E–F) Analysis of expanded-polyglutamine ataxin-3-positive inclusion bodies (IBs) with LC3. The same sets of cells were subjected to immunofluorescence staining with an anti-LC3 (red) antibody in normal (E) and expanded- (F) polyglutamine repeat-expressing cells. (G–H) Recruitment of p62 by ataxin-3 with expanded polyglutamine repeats. Micrographs represent cells transiently transfected with normal (G) ataxin-3(Q28) and expanded- (H) polyglutamine ataxin-3(Q84) plasmids. After 48 h, the cells were subjected to immunofluorescence staining with an anti-p62 (red) antibody. (I–J) Colocalization of 20S proteasome with ataxin-3 containing expanded polyglutamine repeats. Cells that expressed normal (I) and expanded-polyglutamine (J) ataxin-3 were used for immunofluorescence staining with an anti-20S proteasome antibody. A rhodamine-conjugated secondary antibody (red) was used to label ITCH, ubiquitin, LC3, p62 and 20S proteins. The arrows indicate colocalization of ITCH with cytoplasmic inclusion bodies (IBs) containing ataxin-3 with expanded repeats that were also positive for ubiquitin, LC3, p62 and 20S. The overlay images also include 4',6-diamidino-2-phenylindole (DAPI) staining of the cell nuclei. Scale bar, 20 μ m.

Based on the current observations, we anticipated that upregulation of ITCH might provide cytoprotection against misfolded protein-mediated stress. To determine whether ITCH alleviates proteotoxic cell death, we performed a cell viability assay in 293T cells transiently expressing expanded-polyglutamine huntingtin. As shown in Fig. 7, ITCH-transfected cells were significantly more resistant to expanded-polyglutamine-induced cell death than control cells. In contrast, the inactive mutant form of ITCH had no anti-cell-death activity. Depletion of ITCH significantly induced cell death mediated by expanded-polyglutamine proteins. Overall, these findings indicate that the E3 ubiquitin ligase activity of ITCH is essential to cope up under cytotoxicity, and the presence of ITCH is crucial to maintain proteostasis and to alleviate cell death induced by the aggregation of misfolded proteins.

Discussion

In eukaryotic cells, dysfunction of the protein QC mechanism results in accumulation of damaged and misfolded proteins, which causes proteotoxic insults and finally contributes to cellular pathology and neurodegenerative diseases linked with aberrant protein deposition⁵⁰. This study sheds light on how cells maintain the crucial proteome balance during the accumulation of potentially toxic mis-

folded proteins. Multiple lines of previous evidence suggest that selective degradation of misfolded proteins is a possible strategy governing the cellular QC system. Failure of effective degradation of aberrant proteins underlies many diseases such as Huntington's, Parkinson's and age-dependent neurodegenerative diseases⁵¹. Aberrant protein selection and clearance most likely act as key steps in reducing the aggregation of misfolded proteins and enhancing the degradation mediated by decisions of the cellular QC pathway.

In the present study, we analyzed the recruitment and interaction of ITCH with misfolded cytosolic proteins, and similar results were obtained with cytoplasmic inclusions of expanded-polyglutamine proteins. Previously, the HECT ubiquitin ligase Hul5 was shown to participate in the cytosolic protein QC pathway, in which Hul5 recognizes misfolded and damaged proteins that need to be cleared from the dense cellular pool⁵². Here, we observed that endogenous ITCH levels induced after treatment of variety of cellular stresses. ITCH is upregulated under stress conditions; therefore, we hypothesized that this putative E3 ubiquitin ligase may promote QC activity in cells. To test this assumption, we overexpressed GFP-wtCAT and GFP- Δ 9CAT proteins in cells. Surprisingly, we found that ITCH colocalized only with cytoplasmic misfolded inclusions of GFP- Δ 9CAT proteins. Misfolded ITCH-positive GFP- Δ 9CAT inclusions

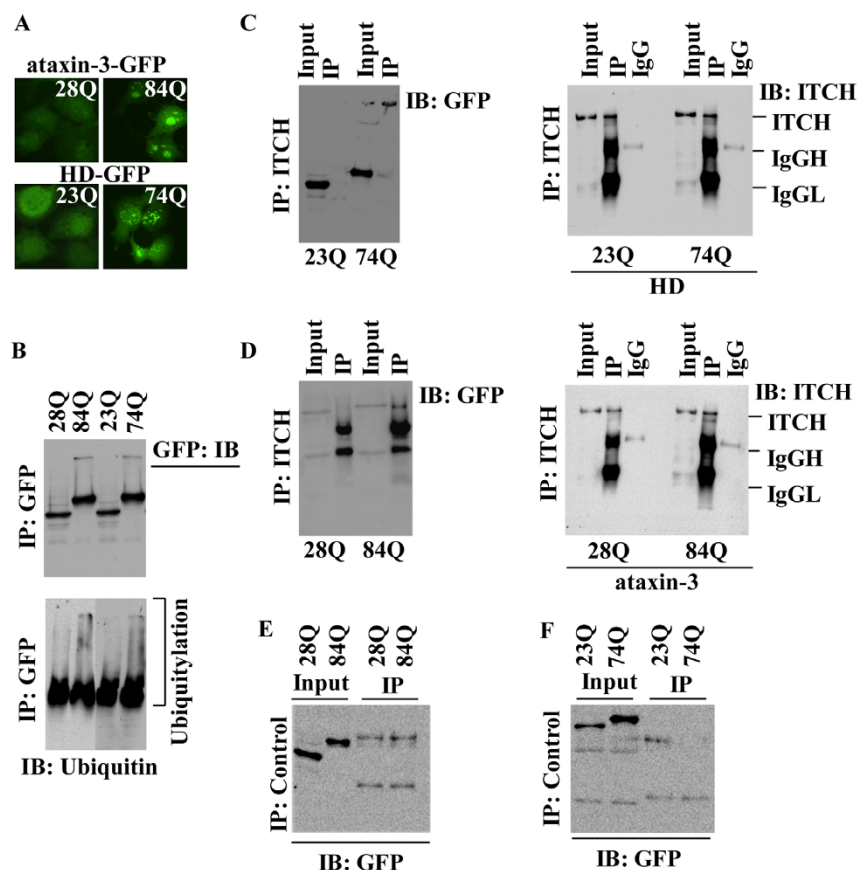


Figure 5 | Interaction of ITCH with expanded polyglutamine proteins. (A–B) Fluorescence micrographs of Cos-7 cells showing the expression and aggregation of expanded-polyglutamine ataxin-3(Q84) (top) and HDQ74 (bottom) proteins (A). Lysates were collected from the same sets of cells and subjected to immunoprecipitation (IP) with the anti-GFP antibody, and the blots were sequentially developed using anti-GFP and anti-ubiquitin antibodies. (C) Cells expressing normal EGFP-HDQ23 and expanded-repeat EGFP-HDQ74 huntingtin were used for coimmunoprecipitation analysis. The cell lysates were pulled down by the anti-ITCH antibody, and the blots were probed with anti-GFP and anti-ITCH antibodies. (D) Transient transfection was used to overexpress normal ataxin-3 (ataxin-3(Q28)) and ataxin-3 with expanded polyglutamine repeats (ataxin-3(Q84)). After 48 h of transfection, the cell lysates were prepared and processed for immunoprecipitation (IP) with the anti-ITCH antibody, and sequentially, the blots were developed using anti-GFP and anti-ITCH antibodies. (E–F) Lysates of cells expressing normal and expanded-polyglutamine versions of ataxin-3 (E) and huntingtin (F) were pulled down by beads only (control), and immunoblotting was performed with an anti-GFP antibody.

were also strongly positive for other components of cellular QC pathways such as ubiquitin, p62/SQSTM1 and 20S proteasome proteins. The parallel functions of Ubiquitin Protein Ligase E3 Component N-Recognin1 (Ubr1) and Sir Antagonist 1 (San1) E3 ubiquitin ligases mediate the degradation of misfolded cytoplasmic proteins and contribute to the cytoplasmic protein QC mechanism⁵³. This study also suggests that few E3 ubiquitin ligases may interact preferentially with cytoplasmic misfolded proteins and further promote their degradation. To confirm the possibility of an interaction of ITCH with a cytosolic misfolded protein (GFP- Δ 9CAT), we performed a detailed coimmunoprecipitation assay and observed that ITCH specifically interacts with GFP- Δ 9CAT. These results further enhance our understanding of the involvement of ITCH with cytosolic misfolded proteins.

In the present study, we tested if ITCH associates with other misfolded proteins. Multiple lines of evidence suggest that the cytoplasmic aggregation of polyglutamine-expanded proteins generate potential toxicity in cellular and organismal models of Huntington's and different forms of ataxia^{54–56}. Therefore, we expressed expanded-polyglutamine huntingtin in cells, and we found that ITCH colocalized with cytoplasmic misfolded inclusions of expanded-polyglutamine huntingtin. We also observed the recruitment of other components of UPS and autophagy pathways to ITCH-positive expanded polyglutamine aggregates. Previously, it

was shown that ataxin-3 is localized in the cytoplasmic compartment of cells and that expression of ataxin-3 with polyglutamine expansions causes cell death^{57–60}. In the present study, we assessed the localization of ITCH with expanded-polyglutamine ataxin-3, and as expected, we observed the recruitment of endogenous ITCH to cytoplasmic inclusions of ataxin-3-expanded polyglutamine proteins. ITCH is widely involved in the polyubiquitination and degradation of various crucial substrates such as mutant glucocerebrosidase, p63, p73 and LATS1 through the proteasome pathway^{61–63}. The interaction between ITCH and ubiquitylated expanded polyglutamine proteins leads to their degradation via both the proteasomal and autophagy pathways. Previously, it was observed that ITCH partially localizes with Deltex and targets it for lysosomal degradation through the formation of K-29 polyubiquitin chains⁶⁴. Interestingly, ITCH also targets the chemokine receptor CXCR4 for ubiquitination at the plasma membrane⁶⁵. Overall, these studies and our current work suggest that ITCH has versatile functions and may have the ability to target a variety of substrates via both proteasomal and lysosomal degradation mechanisms, which may increase interest in the cytoplasmic QC mechanism.

Overall, these results demonstrate that ITCH most likely plays a crucial role in the maintenance of cytoplasmic protein QC events. We speculate that site-specific recruitment and interactions between ITCH and cytosolic misfolded inclusions may suppress the aggrega-

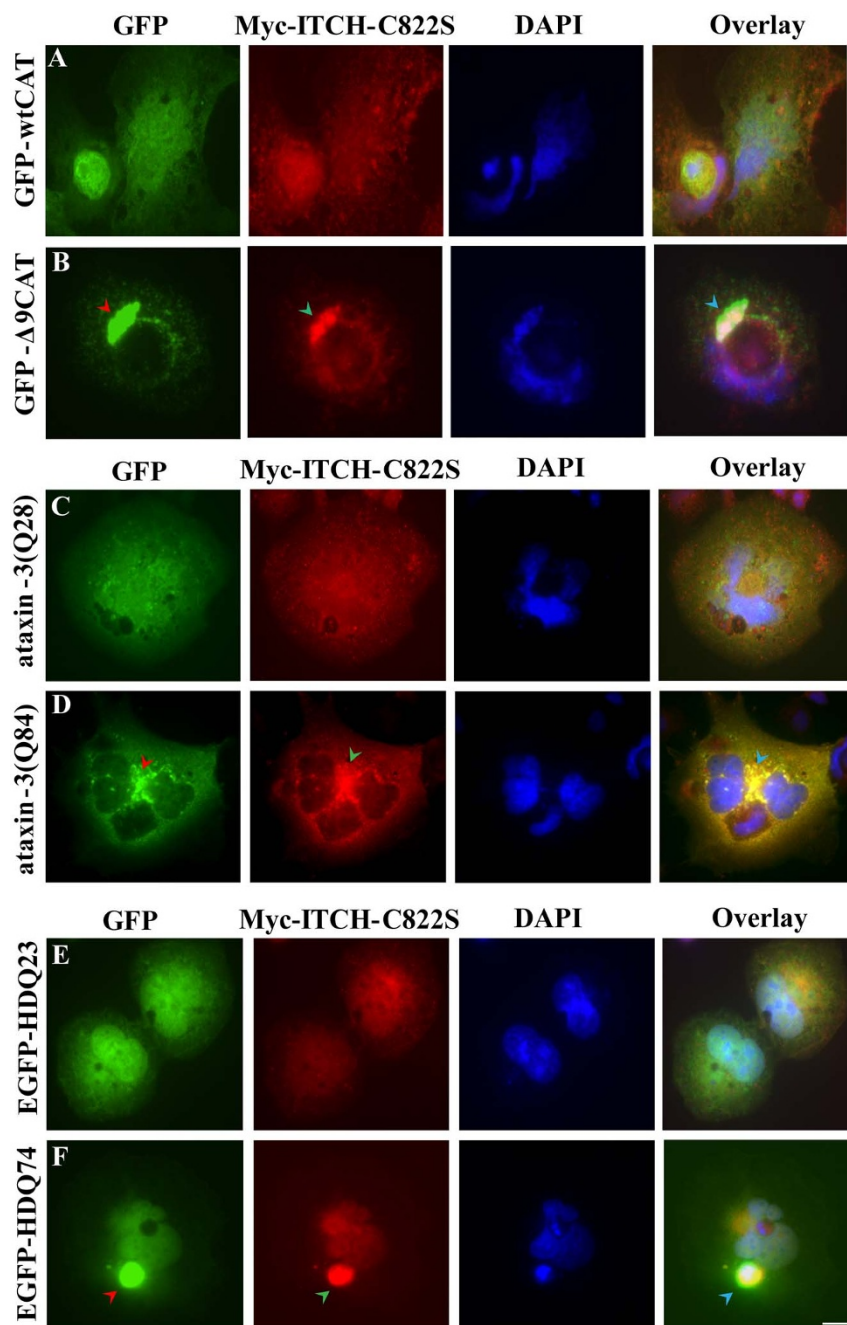


Figure 6 | Redistribution and colocalization of the ITCH mutant with misfolded proteins and expanded polyglutamine proteins. (A–B) The inactive ITCH mutant (Myc-ITCH-C822S) was co-expressed with GFP-wtCAT (A) and GFP- Δ 9CAT (B) constructs. The cells were processed for immunofluorescence staining with an anti-Myc antibody, and nuclear staining was detected using DAPI. Arrows indicate the colocalization of the ITCH-C822S mutant form with cytoplasmic misfolded GFP- Δ 9CAT-protein aggregates. (C–D) As depicted in the figure, normal (C) (ataxin-3(Q28)) and expanded- (D) polyglutamine (ataxin-3(Q84)) ataxin-3 constructs were transiently cotransfected in two-well chamber slides with the ITCH mutant construct. After 48 h of transfection, the cells were subjected to immunofluorescence staining with an anti-Myc antibody, and the nuclei were stained with DAPI. Recruitment of the ITCH mutant form by cytoplasmic inclusion bodies containing ataxin-3 with expanded polyglutamine repeats is indicated by the arrows. (E–F) The expression constructs encoding EGFP-HDQ23 (E) with normal polyglutamine repeats and EGFP-HDQ74 (F) with expanded repeats were expressed with Myc-ITCH-C822S constructs, and the transfected cells were processed for immunofluorescence with an anti-Myc antibody. The arrows indicate the colocalization of the ITCH mutant form with huntingtin HDQ74 cytoplasmic aggregates. A rhodamine-conjugated secondary antibody (red) was used to label Myc-ITCH-C822S. Scale bar, 20 μ m.

tion and toxic effects of the damaged proteins. Consistent with this assumption, we performed an immunoprecipitation assay with ITCH and expanded polyglutamine proteins and found that ITCH preferentially interacts with expanded polyglutamine proteins. Degradation of misfolded proteins in cytoplasmic compartments even at the ribosomal level with the help of E3 ubiquitin ligases

and ribosomal-associated chaperones has been reported^{166,67}; however, the mechanism by which these misfolded proteins are specifically sorted to the cytoplasm for elimination is not known. Our current study identifies ITCH as a critical component of the cytoplasmic protein QC pathway that promotes the degradation of thermally denatured misfolded luciferase protein and expanded-polyglutamine

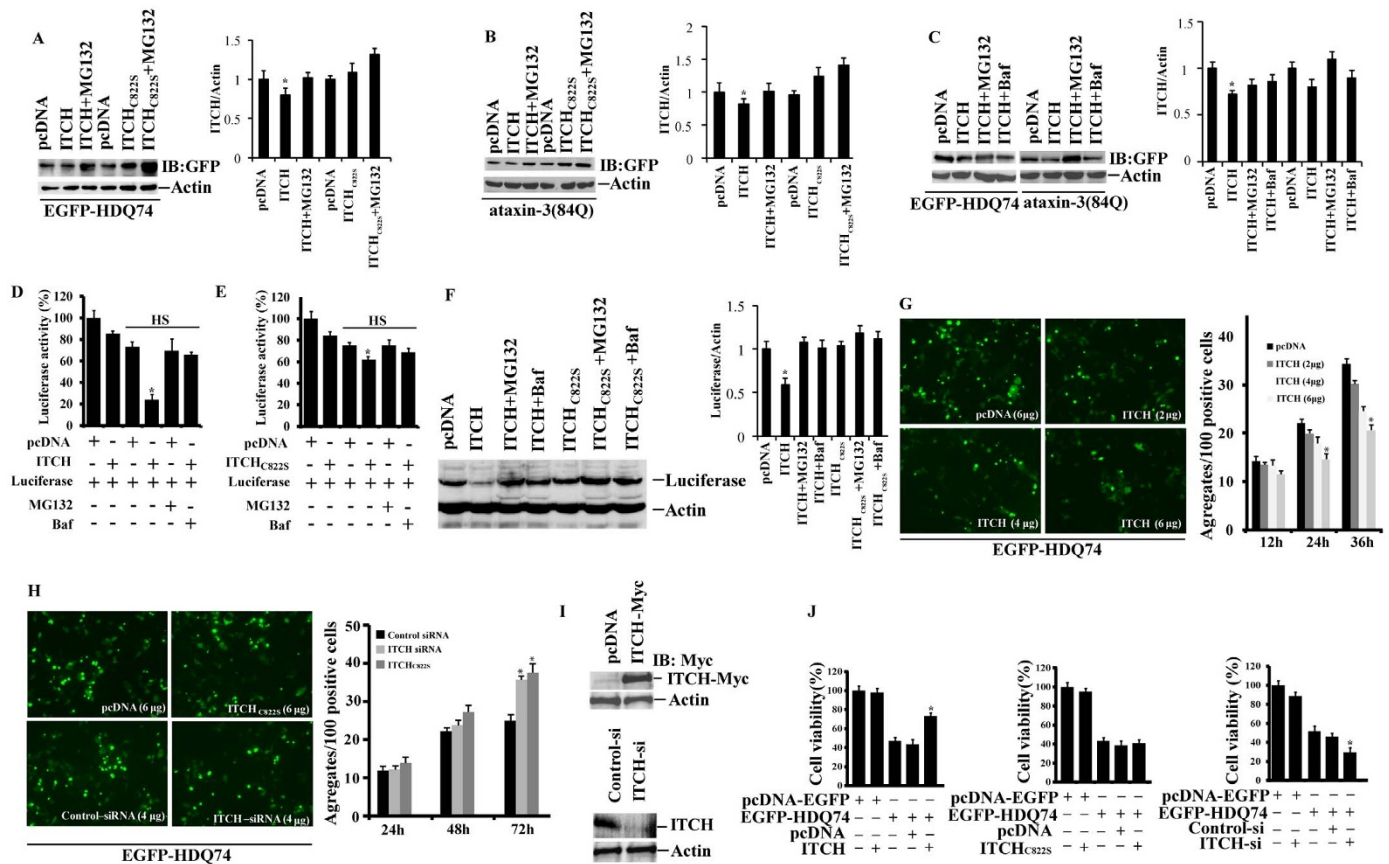


Figure 7 | ITCH rescues polyglutamine aggregation and cytotoxicity. (A–C) Plasmids encoding wild-type ITCH and mutant ITCH-C822S were transiently cotransfected with constructs expressing expanded-polyglutamine huntingtin (EGFP-HDQ74) into Cos-7 cells. After 24 h, the cell extracts were immunoblotted with anti-GFP and anti-actin antibodies (A). Similar experiments were performed with expanded-polyglutamine ataxin-3(Q84), and the blots were probed with anti-GFP and anti-actin antibodies (B). Cells that coexpressed wild-type ITCH with EGFP-HDQ74 and ataxin-3(Q84) proteins were treated with 10 μ M MG132 and 50 nM bafilomycin (Baf). The cell lysates were used for immunoblotting with anti-GFP and anti-actin antibodies (C). Quantification of band intensities collected from three different experiments as shown in section A–C using NIH image analysis software. *, $P < 0.05$ compared with the control. (D–F) ITCH stimulates the degradation of misfolded heat-denatured luciferase. Cos-7 cells were transiently transfected with normal ITCH (D) and mutant ITCH-C822S (E) plasmids together with the firefly luciferase expression construct. After 48 h of transfection, the cells were processed for luciferase activity, and immunoblotting with anti-luciferase and anti-actin antibodies was performed (F). Band intensities were quantified by using NIH image analysis software. *, $P < 0.05$ compared with the control. (G) Cells were transfected with ITCH plasmid as shown in figure, after 48 h, aggregation and quantitation was monitored for EGFP-HDQ74 under a fluorescence microscope. (H) 293T cells were cotransfected with mutant-ITCH-C822S as shown in figure, after 48 h, aggregation was monitored under a fluorescence microscope. Quantitation of EGFP-HDQ74 aggregates in cells transfected with mutant-ITCH-C822S plasmid and ITCH-siRNA oligonucleotides. The results are presented \pm S.D. of three independent experiments each performed in triplicate. *, $P < 0.05$ compared with the control siRNA. (I) Immunoblot analysis of Cos-7 cells transfected with pcDNA and wild-type Myc-ITCH constructs. 293T cells were transiently transfected with control (scrambled siRNA) and ITCH-siRNA oligonucleotides. Cell lysates were prepared and immunoblotted with anti-ITCH and anti-actin antibodies. Full-length blots are presented in Supplementary Figure-S7 (J) Assessment of cytotoxicity mediated by expanded-polyglutamine EGFP-HDQ74 proteins cotransfected with wild-type ITCH or mutant-ITCH-C822S constructs, or ITCH-siRNA oligonucleotides. Cell viability was measured using the MTT assay. Values are the mean \pm S.D. of three independent experiments, each performed in triplicate. *, $P < 0.05$ compared with the control (control siRNA and empty pcDNA) sets.

proteins. This finding suggests that loss of function of ITCH may disturb the cytoplasmic proteostasis produced by many other ubiquitin ligases. To examine this speculation, we knocked down the endogenous level of ITCH and found massive aggregation of expanded-polyglutamine proteins in ITCH-depleted cells. ITCH-specific siRNA-mediated knockdown generated a highly vulnerable condition in cells expressing proteins with expanded polyglutamines. Previously, it was demonstrated that the main components of the cellular QC system (i.e., UPS and autophagy) promote the degradation of exogenous human wild-type, A30P or A53T alpha-synuclein in cells⁶⁸. Similarly, autophagy and UPS also enhance the degradation of expanded polyglutamine proteins in cells and alleviate cytotoxicity^{69,70}. Mutant superoxide dismutase 1 (SOD1) implicated in amyotrophic lateral sclerosis (ALS) motor neuron disease is also

degraded by macroautophagy and the proteasome system⁷¹. Here, we also demonstrated that overexpression of ITCH induces the degradation of misfolded proteins and dramatically reduces the number of aggregates in cells. ITCH upregulation thus provides a cytoprotective response against proteotoxicity generated by expanded-polyglutamine proteins.

A healthy cellular proteome prevents the aggregation of misfolded protein species because proteostasis dysfunction may cause various human diseases⁷². Numerous important questions remain unexplored. How does the cell sense the accumulation of damaged or misfolded proteins in different cellular compartments? Which components of the QC pathways influence the sorting of misfolded proteins in the cytoplasm and why is their recruitment essential at the site of aggregation? Our current study suggests that ITCH most likely



acts as a cytosolic QC E3 ubiquitin ligase that targets abnormal protein inclusions for degradation in the cytoplasm. Therefore, ITCH functionality most likely contributes to the maintenance of an intricate QC system that may be tightly regulated by many other unknown E3 ubiquitin ligases. Emerging studies suggest that even ribosomal-associated chaperones promote cotranslational folding^{73,74} and that the ribosome-bound ligases Rkr1 and Hel2 facilitate cotranslational ubiquitination and assist the nonribosomal ubiquitin ligases Doa 10, Hrd1 and Hul5 to perform cotranslational QC events in cells⁶⁷. These studies support our current findings and speculation that cellular QC function is tightly regulated by a complex network of E3 ubiquitin ligases at various levels in different compartments of a cell, which needs further detailed investigation. Future studies should aim to identify novel QC E3 ubiquitin ligases that will contribute to a better understanding of misfolded protein clearance under normal and pathological conditions and may enable the development of novel therapeutic strategies against aberrant protein aggregation.

Methods

Materials. Bafilomycin, 2-Mercaptoethanol, MG132, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Chloroquine, ITCH specific siRNA oligonucleotides, MISSION® siRNA Universal Negative Control #1 (SIC001) and all cell culture reagents were obtained from Sigma. Dual luciferase reporter gene assay kit was obtained from Promega. Nitroblue tetrazolium salt (NBT), BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt) and Protein G-agarose beads from Roche Applied Science. Lipofectamine®2000 and OptiMEM were obtained from Life Technologies. All monoclonal antibodies: p62/SQSTM1, c-Myc, ITCH, Ubiquitin and actin were obtained from Sigma. Anti-LC3 was purchased from Pierce Antibodies. Monoclonal anti-ubiquitin, anti-luciferase, anti-20S Proteasome and polyclonal anti-GFP were obtained from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were from Amersham Biosciences. Goat anti-rabbit IgG-rhodamine, goat anti-mouse IgG-fluorescein isothiocyanate (FITC), alkaline phosphatase-conjugated anti-rabbit and anti-mouse and IgG were purchased from Vector Laboratories. pcDNA3-EGFP (Addgene 13031), pcDNA3-cmyc (Addgene 16011), Luciferase-pcDNA3 (Addgene 18964), pCINeo-myc-Itch/2859 (Addgene 11427), pCINeo-myc-Itch (C822S)/2860 (Addgene 11428), pEGFP-C1-Ataxin3Q28 (Addgene-22122) and pEGFP-C1-Ataxin3Q84 (Addgene-22123) were purchased from Addgene.

Cell culture, transfection, and cell viability assay. Cos-7 cells and 293T cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum with 100 U/ml penicillin and 100 mg/ml streptomycin. One day prior to the transfection, cells were plated into 6-well, 24 well and 96 well tissue culture plates at a subconfluent density for different experiments. Cells were transiently transfected with expression constructs using the Lipofectamine®2000 reagent according to the manufacturer's instructions. The transfection efficiency was about 70–80%. Amounts of construct DNA in separate transfection were adjusted by control or empty plasmid. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to measure cell viability by MTT assay.

Co-immunoprecipitation experiment and immunoblotting. Cos-7 cells were transiently transfected with various plasmids for forty eight hours. After transfection cells were collected and washed with phosphate-buffer saline, lysed with radioimmune precipitation assay buffer (10 mM Hepes (pH 7.4), 2.5 mM EGTA, 10 mM EDTA, 150 mM NaCl, 5 mM Na₂P₂O₇, 10 mM NaF, 0.1 mM Na₂VO₅, 0.1 mg/ml Aprotinin, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS). Cell lysates were briefly sonicated and centrifuged for 10 minutes at 15,000 × g at 4°C. The total cell lysate or the immunoprecipitated samples were separated through SDS-polyacrylamide gel electrophoresis and used for immunoblotting as described elsewhere²⁵. All primary antibodies were used in 1:1000 dilutions, overnight incubations at 4°C.

RNA interference (RNAi) experiments, aggregate counting, and reporter gene assay. 293T Cells were plated into 6-well tissue culture plates and on the following day cells were transiently cotransfected with MISSION® siRNA Universal Negative Control or ITCH-siRNA oligonucleotides and other plasmids, details as described earlier²⁶. Few plates were transiently cotransfected with ITCH, ITCH mutant plasmid (Myc-ITCH-C822S) and various expanded polyglutamine constructs. After forty-eight hours, cells were collected and processed for immunoblotting experiment and blots were developed with anti-GFP, anti-ITCH, anti-Myc and anti-actin. Similar sets of cells were used for cell viability assay and aggregate counting, cells remain more than one aggregate were considered to have a single big inclusion. Fluorescence microscope (~500 transfected cells in each case) was used to analyze and count expanded polyglutamine aggregate formation. For luciferase assay, cells were transiently transfected with various plasmids and incubate in a normal CO₂ incubator or heat stressed at 43°C for 30 minutes and then to the normal CO₂ incubator for

1 hour. In few experiments cells were pre-treated with MG132 (10 μM) or bafilomycin (50 nM) just prior to heat stress. Cells were then collected and processed for measuring luciferase activity according to the manufacturer's instructions from Promega (Dual luciferase reporter gene assay kit).

Immunofluorescence technique. Cells grown in chamber slides were transiently transfected with different constructs for forty-eight hours. Post transfection, cells were washed three times with phosphate-buffer saline (PBS) and used 4% paraformaldehyde in PBS for their 15 minutes fixation. Triton X-100 (0.5%) in PBS was used for permeabilization of fixed cells for 5 minutes, washed them for 4 times in PBS and then blocked with 5–10% normal goat serum in Tris-buffered saline with Tween 20 (TBST) for 60 minutes. Primary antibodies (1:500 dilutions) were used for overnight incubation at 4°C. After incubation cells were extensively washed with TBST and incubated with rhodamine-conjugated and fluorescein isothiocyanate-conjugated antibodies for 60 minutes, washed them with TBST for several times and mounted with antifade solution. Nuclear staining was carried out with 4',6-diamidino-2-phenylindole (DAPI) staining and images were acquired using fluorescence microscope.

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Author contributions

D.C., A.U., A.A., V.J. and A.M. executed the experiments. A.M. designed the experiments and wrote the manuscript. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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