Cell

Autophagic Clearance of PolyQ Proteins Mediated by Ubiquitin-Atg8 Adaptors of the Conserved CUET Protein Family

Kefeng Lu,[1] Ivan Psakhye,[1] and Stefan Jentsch[1,*]
[1]Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany
*Correspondence: jentsch@biochem.mpg.de
http://dx.doi.org/10.1016/j.cell.2014.05.048

SUMMARY

Selective ubiquitin-dependent autophagy plays a pivotal role in the elimination of protein aggregates, assemblies, or organelles and counteracts the cytotoxicity of proteins linked to neurodegenerative diseases. Following substrate ubiquitylation, the cargo is delivered to autophagosomes involving adaptors like human p62 that bind ubiquitin and the autophagosomal ubiquitin-like protein Atg8/LC3; however, whether similar pathways exist in lower eukaryotes remained unclear. Here, we identify by a screen in yeast a new class of ubiquitin-Atg8 adaptors termed CUET proteins, comprising the ubiquitin-binding CUE-domain protein Cue5 from yeast and its human homolog Tollip. Cue5 collaborates with Rsp5 ubiquitin ligase, and the corresponding yeast mutants accumulate aggregation-prone proteins and are vulnerable to polyQ protein expression. Similarly, Tollip depletion causes cytotoxicity toward polyQ proteins, whereas Tollip overexpression clears human cells from Huntington's disease-linked polyQ proteins by autophagy. We thus propose that CUET proteins play a critical and ancient role in autophagic clearance of cytotoxic protein aggregates.

INTRODUCTION

Protein misfolding and aggregation can be cytotoxic for cells and may lead to cell death, but organisms respond by evoking several containment measures. Molecular chaperones are expressed to repair protein folding or to disentangle protein aggregates or prevent their formation (Kim et al., 2013). In cases in which misfolded or aggregated proteins persist, degradation via the proteasome or the lysosome is the typical means to clear toxic components from cells (Kirkin et al., 2009b; Stefani and Dobson, 2003). Selective degradation of soluble proteins is usually conducted by the ubiquitin-proteasome system (UPS) (Ravid and Hochstrasser, 2008). By contrast, tightly folded proteins or protein aggregates, which are unable to traverse the narrow openings of the proteasome (Finley, 2009; Piwko and Jentsch, 2006) and are hence resistant to proteasomal degradation, may accumulate in cells as ubiquitylated species (Bence et al., 2001). Several studies suggested that such ubiquitin-modified species or aggregates are recognized by the selective macroautophagy pathway (here termed autophagy) (Mizushima et al., 2011). Notably, this alternative ubiquitin-dependent degradation pathway is used to eliminate not only aberrant proteins or complexes, but also superfluous or damaged cellular structures like mammalian midbodies, organelles, or even bacteria, and is hence critical for innate immunity (Klionsky, 2007; Levine et al., 2011; Pohl and Jentsch, 2009). Autophagy is further relevant to human health and disease by the finding that protein aggregates are a common feature in a number of neurodegenerative disorders (Levine and Kroemer, 2008). For example, tau-containing neurofibrillary tangles accumulate in Alzheimer's disease and α-synuclein in Lewy bodies in Parkinson's disease, and aggregates of poly-glutamine (polyQ) mutant variants of the protein huntingtin are formed in patients with Huntington's disease (Choi et al., 2013). Indeed, autophagy appears abnormal in many neurodegenerative diseases, and inactivation of autophagy can lead to neurodegeneration associated with an accumulation of ubiquitylated structures (Hara et al., 2006).

Selective autophagy of ubiquitylated cargo is achieved by the action of specific adaptors that connect the ubiquitin system with the autophagy pathway (Kraft et al., 2010; Rogov et al., 2014). Known adaptors are, e.g., human p62 (also known as SQSTM1) and its relative NBR1, which both harbor a UBA domain for ubiquitin-conjugate binding and a distinct binding site termed AIM (Atg8-interacting motif) or LIR (LC3-interacting region) for the autophagosomal protein Atg8 (LC3) (Kirkin et al., 2009a; Kraft et al., 2010; Pankiv et al., 2007). Atg8 is structurally similar to ubiquitin but becomes conjugated to the lipid phosphatidylethanolamine of the autophagosomal membrane through a ubiquitin-like conjugation system (Ohsumi, 2001). Notably, Atg8 is not only required for productive autophagosome formation, but also serves as a central docking module for autophagic cargos prior to their delivery to lysosomal degradation via the autophagy pathway (Mizushima et al., 2011). Thus, ubiquitin-Atg8 adaptors such as p62 are fundamentally important for autophagy selectivity as they recognize the substrates directly. Indeed, human p62 and to a lesser extent the related protein NBR1 have been shown to associate with protein assemblies and aggregates, including those linked to neurodegenerative diseases (Kirkin et al., 2009a). On the other hand, only a weak

enhancement of ubiquitylated aggregates was observed in p62 or NBR1 knockout mice or cells, suggesting that alternative or additive pathways might exist (Komatsu et al., 2007). Moreover, although the central components of the autophagy pathway have been initially delineated in *Saccharomyces cerevisiae* (Mizushima et al., 2011) and are well conserved from yeast to mammals, so far no ubiquitin-Atg8 adaptor has been described in yeast (Rogov et al., 2014). In fact, even whether ubiquitin-dependent cargo selection for macroautophagy exists in lower eukaryotes is unclear (Kraft et al., 2010).

Because ubiquitin-Atg8 adaptors lie at the heart of autophagy selectivity, we embarked on a biochemical screen in *S. cerevisiae* for proteins that bind yeast Atg8 and ubiquitin. Using a mass spectrometric approach, we identified Cue5, a yeast protein harboring a ubiquitin-binding CUE domain (Kang et al., 2003; Prag et al., 2003), as a ubiquitin-Atg8 adaptor and showed that it indeed functions in ubiquitin-dependent autophagy. This discovery helped us to identify the human CUE-domain protein Tollip as a functional Cue5 homolog. Notably, these related adaptors, which we termed CUET proteins (CUE-domain targeting adaptors; Cue5-Tollip proteins), are crucial for autophagic removal of protein aggregates, such as proteins derived from expanded polyQ isoforms of huntingtin. Our findings thus suggest that ubiquitin-Atg8 adaptors of the CUET protein family play an evolutionarily conserved role in autophagy, specifically by combating the cytotoxicity caused by aggregation-prone proteins.

RESULTS

Accumulation of Ubiquitin-Protein Conjugates in Yeast Autophagy Mutants

Given that the ubiquitin system and the basic autophagy machinery are conserved form yeast to mammals (Rogov et al., 2014), we found it reasonable to assume that ubiquitin-Atg8 adaptors may also exist in yeast. To search for possible ties between the ubiquitin and the autophagy system in *S. cerevisiae*, we first asked whether ubiquitin conjugates accumulate in yeast autophagy pathway mutants under conditions when autophagy is activated. Because autophagy is universally induced upon starvation (Mizushima et al., 2011), we shifted yeast cells into a synthetic minimal medium lacking nitrogen (SD-N medium; see Experimental Procedures) and followed the pattern of cellular ubiquitin conjugates using ubiquitin-specific antibodies. We noticed that the levels of ubiquitin conjugates, as identified by immunoblotting, decreased rapidly in wild-type (WT) cells after starvation (Figure 1A). The observed decrease affected predominantly high molar mass ubiquitin conjugates, suggesting that the phenomenon may be more specific for polyubiquitylated proteins. Notably, when we used mutants deficient in the autophagosomal ubiquitin-like protein Atg8 (*Δatg8*), starvation-induced loss of ubiquitin conjugates was strongly reduced (Figure 1A). Moreover, ubiquitin conjugates also remained high in starved mutants lacking the autophagy-activating kinase Atg1 (*Δatg1*), the E1-like enzyme Atg7 needed for Atg8 lipidation (*Δatg7*), and also in cells deficient in peptidase Pep4 (*Δpep4*) of the vacuole (yeast lysosome-related organelle) (Figures S1A and S1B available online). When we activated autophagy by blocking the TOR (target of rapamycin) pathway by adding rapamycin to the medium (Noda and Ohsumi, 1998), the level of ubiquitin conjugates again dropped in WT cells, but not in autophagy mutants (Figures 1B and S1C). By contrast, no accumulation of ubiquitin conjugates was detectable in mutants lacking Atg11 and Atg19 (*Δatg11*, *Δatg19*), two proteins needed for the autophagy-related cytoplasm-to-vacuole targeting (Cvt) pathway (Feng et al., 2014) (Figure S1A). From this finding, we infer that the observed effect on ubiquitin conjugates is specific for the central autophagy pathway (macroautophagy) that acts, for example, on protein aggregates. Indeed, we noticed that the level of insoluble ubiquitin conjugates that accumulate upon elevated growth temperatures in WT cells (Figure 1C; P, pellet fraction) was much higher when autophagy was blocked (*Δatg8*; Figure 1C), which was not observed in mutant cells lacking the protein Atg11 (*Δatg11*) (Figure S1D). From these lines of evidence we thus conclude that the ubiquitin and autophagy pathways are functionally linked also in yeast and that autophagy may be utilized to clear ubiquitylated protein aggregates from cells.

Yeast Cue5 Functions as Ubiquitin-Atg8 Adaptor

Because Atg8 from mammalian cells interacts with ubiquitin conjugates via ubiquitin-Atg8 adaptors (Rogov et al., 2014), we next asked whether yeast Atg8 associates with ubiquitin conjugates as well. In fact, when we immunoprecipitated Atg8 (HA-epitope-tagged Atg8; HA-Atg8) from yeast cell extracts, we co-isolated ubiquitin conjugates (Figure 2A). In an attempt to screen for putative ubiquitin-Atg8 adaptors, we immunoprecipitated proteins using HA-specific antibody from starved WT and HA-Atg8-expressing cells and identified the bound proteins by mass spectrometry (see Figure S2A and Methods). As expected, we detected peptides for Atg8 itself and its ubiquitin-like conjugation machinery (Atg7, Atg3) specifically in the sample of HA-Atg8-expressing cells (Figure S2B). Interestingly, also enriched in this sample were peptides corresponding to the ubiquitin ligase Rsp5 (see below) and Cue5, a protein bearing a putative ubiquitin-binding CUE domain (Kang et al., 2003; Prag et al., 2003).

As Cue5 also possesses a putative Atg8-interacting motif (AIM) close to its carboxyl (C) terminus (Figure 2B), we asked whether the previously uncharacterized yeast protein binds Atg8. Indeed, as judged by an immunoprecipitation experiment, Cue5 and Atg8 interact in vivo, particularly in starved yeast cells (Figure 2C). Furthermore, we detected the preferential binding of Cue5 to the lipidated form of Atg8 (Atg8-PE) (Figures 2D and S2C). By contrast, no interaction was observed between Cue5 and the protein Atg11 linked to the Cvt pathway (Figure S2D). Interestingly, in starved cells, binding of Atg8 to ubiquitin conjugates was largely dependent on Cue5 (Figure S2E), suggesting that Cue5 may indeed be a key ubiquitin-Atg8 adaptor. Importantly, binding of Atg8 to Cue5 is direct and is mediated by the C-terminal AIM, as indicated by glutathione S-transferase (GST) pull-down experiments using purified GST fusions of WT Cue5 and of variants in which critical residues of the AIM sequence had been altered (single replacement: Cue5-W373A, Cue5-L376A; complete replacement: Cue5-W373A, Q374A, P375A, L376A = Cue5-AIM^{mut}; Figure 2E).

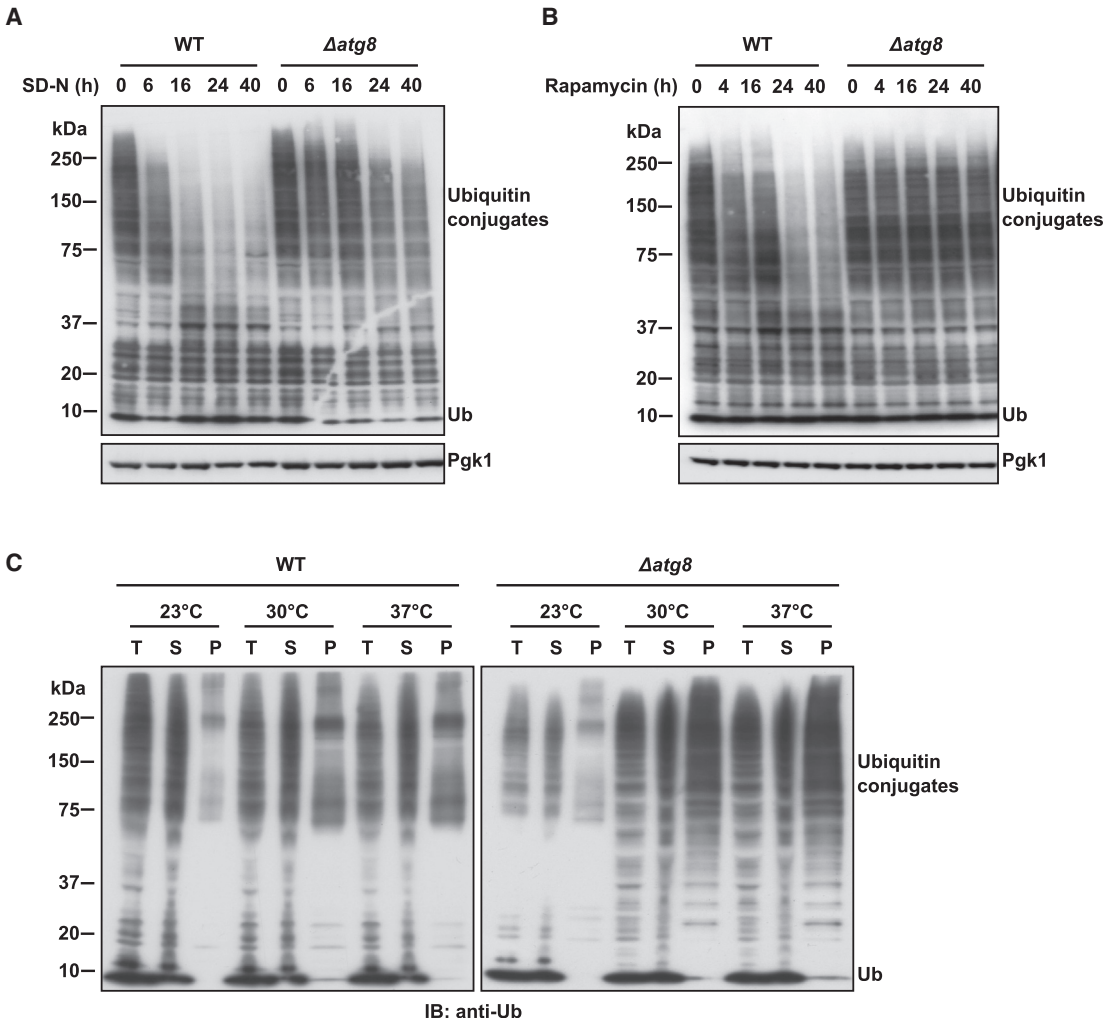


Figure 1. Ubiquitin Conjugates Accumulate in Autophagy-Deficient Cells

(A) Ubiquitin conjugates are degraded by the autophagy pathway upon starvation, and degradation is blocked in cells lacking Atg8. Wild-type (WT) and Atg8-deficient (*Δatg8*) cells were subjected to starvation in synthetic minimal medium lacking nitrogen (SD-N) for indicated times (h). Cell lysates were probed for ubiquitin (Ub) conjugates by immunoblotting using anti-ubiquitin antibodies (P4D1). Immunoblot against Pgk1 serves as loading control.

(B) Activation of autophagy by rapamycin promotes the degradation of ubiquitin conjugates.

(C) Insoluble ubiquitin conjugates accumulate in autophagy-deficient cells. WT and Atg8-deficient (*Δatg8*) cells were cultured at different temperatures and fractionated by centrifugation, and ubiquitin conjugates were detected in total cell lysate (T), soluble (S), and pellet (P) fractions. 1% of the T and S fractions and 20% of the isolated P fraction were loaded onto the gel.

See also Figure S1.

We confirmed that Cue5 also binds free His-tagged ubiquitin in vitro (Figure S2F) and endogenous ubiquitin-conjugates in vivo (Figure 2F). Ubiquitin binding of Cue5 is, in fact, mediated by its CUE domain, as substitutions of amino acid residues (Cue5-F109A, P110A; Cue5-L135A, L136A) crucial for ubiquitin binding of the related CUE domain of the yeast endocytic pathway protein Vps9 (Shih et al., 2003) abolished Cue5 ubiquitin-conjugate binding in vivo (Figure 2F). Notably, the fusion of the Cue5 CUE domain (residues 89–190) to GST interacted with isolated lysine-48 (K48) and lysine-63 (K63)-linked polyubiquitin chains (Figure S2G), suggesting that Cue5 might not distinguish much between various types of ubiquitin modifications.

Next, we tested whether Cue5 is important for the clearance of ubiquitin conjugates in starved yeast cells similar to Atg8. Indeed, we found that starvation-induced decay of ubiquitin conjugates was strongly reduced in cells lacking Cue5 (*Δcue5*) (Figure 2G). Because this defect in ubiquitin-conjugate clearance also occurred in cells expressing (as only source of Cue5) Cue5 variants with functionally compromised CUE (Cue5-L135A, L136A = Cue5-CUE^{mut}) and AIM (Cue5-AIM^{mut}) motifs (Figure 2G), we deduce that both ubiquitin-conjugate binding and Atg8 interaction are required for Cue5 functionality. Furthermore, Cue5 appears to act specifically at the stage of ubiquitin-conjugate recognition, as autophagy itself (indicated by GFP-Atg8

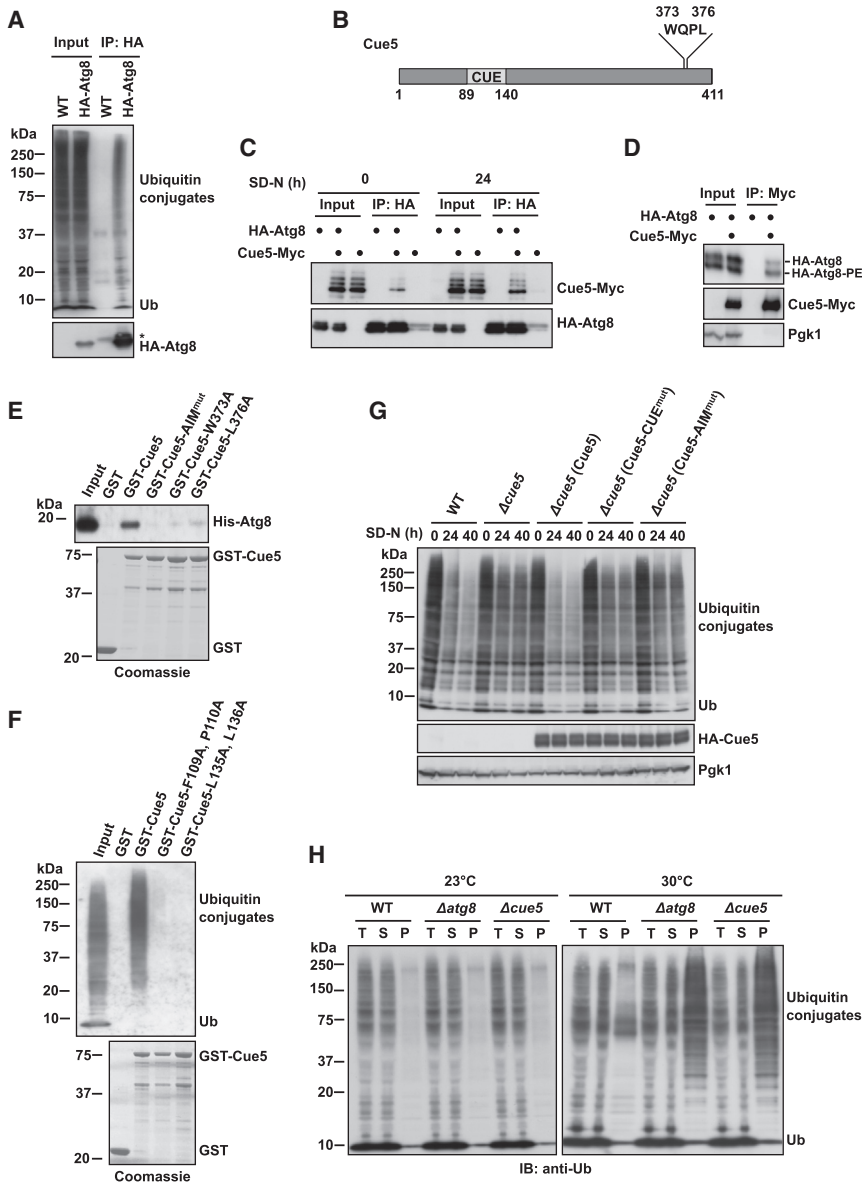


Figure 2. Identification of Cue5 as Ubiquitin-Atg8 Adaptor of *S. cerevisiae*

(A) Atg8 binds ubiquitin conjugates. Cells expressing N-terminally HA-tagged Atg8 were subjected to starvation in SD-N media for 12 hr before immunoprecipitation with anti-HA affinity matrix. Proteins of the extract (Input) and precipitated proteins (IP) were analyzed by immunoblotting with anti-HA and anti-ubiquitin (P4D1) antibodies. Free ubiquitin (Ub) is indicated. The asterisk denotes the IgG light chain.

(B) Schematic representation of yeast Cue5 containing the ubiquitin-binding CUE domain (aa 89–140) and a putative Atg8-interacting motif (AIM, aa 373–376).

(C) Cue5 interacts with Atg8 in vivo, and this interaction is increased by starvation-induced autophagy. Atg8 and Cue5 were HA and Myc epitope tagged, respectively, and their interaction was detected by coimmunoprecipitation before and after starvation in SD-N medium.

(D) Cue5 preferentially interacts with the lipidated form of Atg8 in vivo. Coimmunoprecipitation assay conducted similar to (C). Lipidated Atg8 (Atg8-PE) resolved by SDS-PAGE on a 13.5% polyacrylamide gel containing 6M urea.

(E) Cue5 interacts with Atg8 directly via its putative AIM. GST pull-down assays were carried out using recombinant His-tagged Atg8 and GST fusions of WT Cue5, or Cue5-AIM mutants (Cue5-W373A; Cue5-L376A; Cue5-AIM^{mut} = Cue5-W373A, Q374A, P375A, L376A). Immunoblot against anti-His(Atg8) and Coomassie-blue-stained gel are shown.

(F) Amino acid replacements in the CUE domain of Cue5 abolish its ubiquitin-conjugate-binding ability. GST pull-down assays were performed using yeast whole-cell extracts and GST fusions of WT Cue5 or Cue5 variants (Cue5-F109A, P110A; Cue5-L135A, L136A).

(G) Cue5 mediates autophagic degradation of ubiquitin conjugates through binding to ubiquitin and Atg8. WT or respective CUE-domain mutant (Cue5-CUE^{mut} = Cue5-L135A, L136A) and AIM (Cue5-AIM^{mut}) mutant variants of Cue5 were expressed in Cue5-deficient cells (*Δcue5*), and ubiquitin conjugates were monitored by immunoblotting (as in Figure 1A) after starvation in SD-N medium.

(H) Insoluble ubiquitin conjugates accumulate at elevated temperatures in Cue5-deficient (*Δcue5*) cells as in autophagy-deficient (*Δatg8*) cells. Cell extracts were fractionated and ubiquitin conjugates detected in total cell lysate (T), soluble (S), and pellet (P) fractions (as in Figure 1C).

See also Figure S2.

degradation; Klionsky et al., 2012) is not affected when Cue5 is absent (Figure S2H). In addition, other selective autophagy-related pathways do not seem to be affected in Cue5-deficient cells, such as the Cvt pathway (Figure S2I), mitophagy (Figure S2J), pexophagy (Figure S2K), and ribophagy (Figure S2L). Importantly, Cue5-dependent autophagic clearance seems to be specifically relevant for aggregated proteins because insoluble ubiquitin conjugates strongly accumulate upon elevated temperatures (30°C compared to 23°C) in mutants deficient in Cue5 as they do in Atg8-deficient cells (Figure 2H). Because Cue5 protein levels are barely affected by higher temperature but decrease upon starvation through vacuolar degradation (Figure S2M), heat-stress-induced autophagy may target less cargo than starvation-induced autophagy. From all of these findings, we thus conclude that Cue5 indeed connects the ubiquitin pathway to autophagy in yeast by functioning as a ubiquitin-Atg8 adaptor analogous to p62 in mammals.

Cue5 Targets Aggregation-Prone Proteins for Autophagy

To determine what type of proteins are subject to ubiquitin- and Cue5-dependent autophagy, we isolated ubiquitin conjugates from starved WT and Cue5-deficient cells and compared the purified proteins by a SILAC-based mass spectrometry protocol

(Mann, 2006; Figure 3A and Experimental Procedures). We focused on 24 proteins most strongly enriched in the sample from Cue5-deficient cells (Figure 3B; proteins are listed in Figure S3A) and tested them individually for Cue5-dependent degradation. After expression of these proteins as GFP fusions in cells and induction of autophagy by starvation, we noticed in all cases an accumulation of a stable (vacuolar protease-resistant) GFP fragment (Klionsky et al., 2012), as is typical for autophagy substrates (Figures 3C and S2H and data not shown). Indeed, the production of stable GFP fragments depended on the presence of Atg8 and largely also Cue5 (Figure 3C), demonstrating that the tested proteins are bona fide substrates of a Cue5- and Atg8-dependent autophagy pathway.

The identified Cue5 substrates are primarily cytosolic proteins of diverse cellular functions and share no obvious common likenesses (Figure S3A). However, because aggregated proteins are frequently targeted to autophagy (Rogov et al., 2014), we asked whether the identified Cue5 substrates are perhaps aggregation prone. Indeed, for some of the tested Cue5 substrates (as GFP fusions), we found that they tend to aggregate already at normal growth temperature (30°C), as indicated by the presence of the proteins in the insoluble pellet fraction of cell extracts after centrifugation (Fang et al., 2011; Figure S3B). By contrast, non-aggregation-prone proteins such as GFP-tagged Ubc9 ($Ubc9^{WT}$-GFP), ubiquitin, or SUMO, used previously in similar assays (Kaganovich et al., 2008) (Figures S3B and S3C), were virtually completely soluble, whereas a few Cue5 substrates (e.g., Ent2 and Gvp36) only mildly aggregated at 30°C (Figure S3B).

We next compared the solubility of proteins in WT and Cue5-deficient cells, in which we induced protein aggregation by growing them at a higher temperature (37°C instead of 23°C). Notably, when we analyzed an only mildly aggregation-prone Cue5 substrate like Gvp36 (as a GFP fusion), a fraction of the protein accumulated in the pellet fraction of WT cells, but only if they were grown at the higher temperature (Figure 3D). As this fraction considerably increased in Cue5-deficient cells, we conclude that the tested aggregated protein is normally targeted to Cue5-dependent autophagy. Moreover, the extent of Gvp36 autophagic Cue5-dependent degradation increased substantially at elevated temperatures, as judged from a GFP-cleavage assay (Figure 3E). To extend this finding, we also used $Ubc9^{ts}$-GFP (a mutant variant of soluble $Ubc9^{WT}$-GFP), which is known to aberrantly fold and aggregate at 37°C (Kaganovich et al., 2008). As this protein aggregated at 37°C in Cue5-deficient cells slightly more than in WT (albeit not nearly as markedly as for Gvp36), a small fraction of this mutant protein seems to be a Cue5 substrate as well (Figure S3C). By contrast, $Ubc9^{WT}$-GFP, ubiquitin, or SUMO remained soluble in Cue5-deficient cells even when grown at the higher temperature (Figures 3D and S3C). Furthermore, we did not observe interaction between Cue5 and ubiquitin-proline-β-galactosidase, a well-characterized soluble proteasome substrate (Bachmair et al., 1986) despite its high rate of polyubiquitylation (Figure S3D). Together, these data thus suggest that ubiquitin- and Cue5-dependent autophagy act prominently on aggregation-prone proteins under cellular conditions when protein aggregation otherwise occurs.

Ubiquitylation Pathway Connected to Cue5-Dependent Autophagy

Because Rsp5 ubiquitin ligase (Rotin and Kumar, 2009) was found in the fraction of Atg8-copurifying proteins (see above) and Ubc4 and Ubc5, two nearly identical ubiquitin-conjugating enzymes (Seufert and Jentsch, 1990), were among the potential Cue5 substrates (Figure S3A), we speculated that these enzymes of the ubiquitin pathway might be functionally linked to Cue5-dependent autophagy. Intriguingly, starvation-induced autophagy of several Cue5 substrates was substantially inhibited in cells expressing defective Rsp5 (*rsp5-2*, conditional mutant of the essential gene *RSP5*) and also in cells lacking Ubc4, Ubc5, or both enzymes (Figures 4A, 4B and data not shown). This suggests that a sizable fraction of Cue5-dependent autophagy substrates receives their ubiquitin mark by the activity of Ubc4, Ubc5, and Rsp5 enzymes. In fact, by analyzing two Cue5 substrates (Tma19 and Fpr1 expressed as GFP fusions) in cells expressing His-tagged ubiquitin, we found that their ubiquitylation depended on active Rsp5 (Figure 4C) and that binding of Cue5 to these substrates was strongly reduced when Rsp5 was defective (Figure 4D). Nevertheless, autophagy of some proteins (e.g., the ribosomal protein Rpp2b) occurred independently of Rsp5 (Figure S4A), indicating that ubiquitin ligases other than Rsp5 might act in the Cue5 pathway as well.

Interestingly, immunoprecipitation of Rsp5 co-isolated Cue5 (Figure 4E), suggesting that ubiquitin-conjugate formation and ubiquitin-conjugate recognition are intimately coupled. We also noticed that Cue5 itself is ubiquitin modified (Figure S4B) and that this depends on Rsp5 as well (Figure S4C). Indeed, the autophagy adaptor Cue5 itself is subject to starvation-induced autophagy (similar to mammalian p62), which is mediated by Atg8 and also largely by Rsp5 (Figures 4F and S2M). Notably, the autophagy pathway is still active in the absence of Rsp5 activity (as monitored by GFP-Atg8 cleavage), demonstrating that Rsp5 is not required for the core activity of the autophagy pathway (Figure S4D). Complementing the results of our SILAC experiment (Figure S3A), we found that Ubc4 and also Rsp5 are substrates of Atg8- and Cue5-dependent starvation-induced autophagy (Figures S4E and S4F). We thus infer that Ubc4/Ubc5 and Rsp5 are directly involved in earmarking proteins for autophagy upon starvation and that a fraction of the ubiquitylation and autophagy machineries is targeted conjointly with genuine autophagy substrates to autophagosomes and vacuolar degradation.

Rsp5 and Cue5 Promote the Clearance of Huntingtin-Derived PolyQ Proteins

Owing to the finding that the spectrum of Cue5 substrates is enriched in aggregation-prone proteins (Figures 3D and S3B) and that insoluble proteins accumulate in Cue5-deficient cells (Figure 2H), we speculated that the Cue5-dependent selective autophagy pathway might clear cells from aggregates. Particularly well-studied aggregates are those caused by Huntington's-disease-associated cytotoxic variants of the protein huntingtin that bear expanded tracks of glutamine residues (polyQ) (Choi et al., 2013). We took advantage of two model substrates encompassing the polyQ region expressed from exon-1 of the huntingtin-encoding gene: an aggregation-prone variant

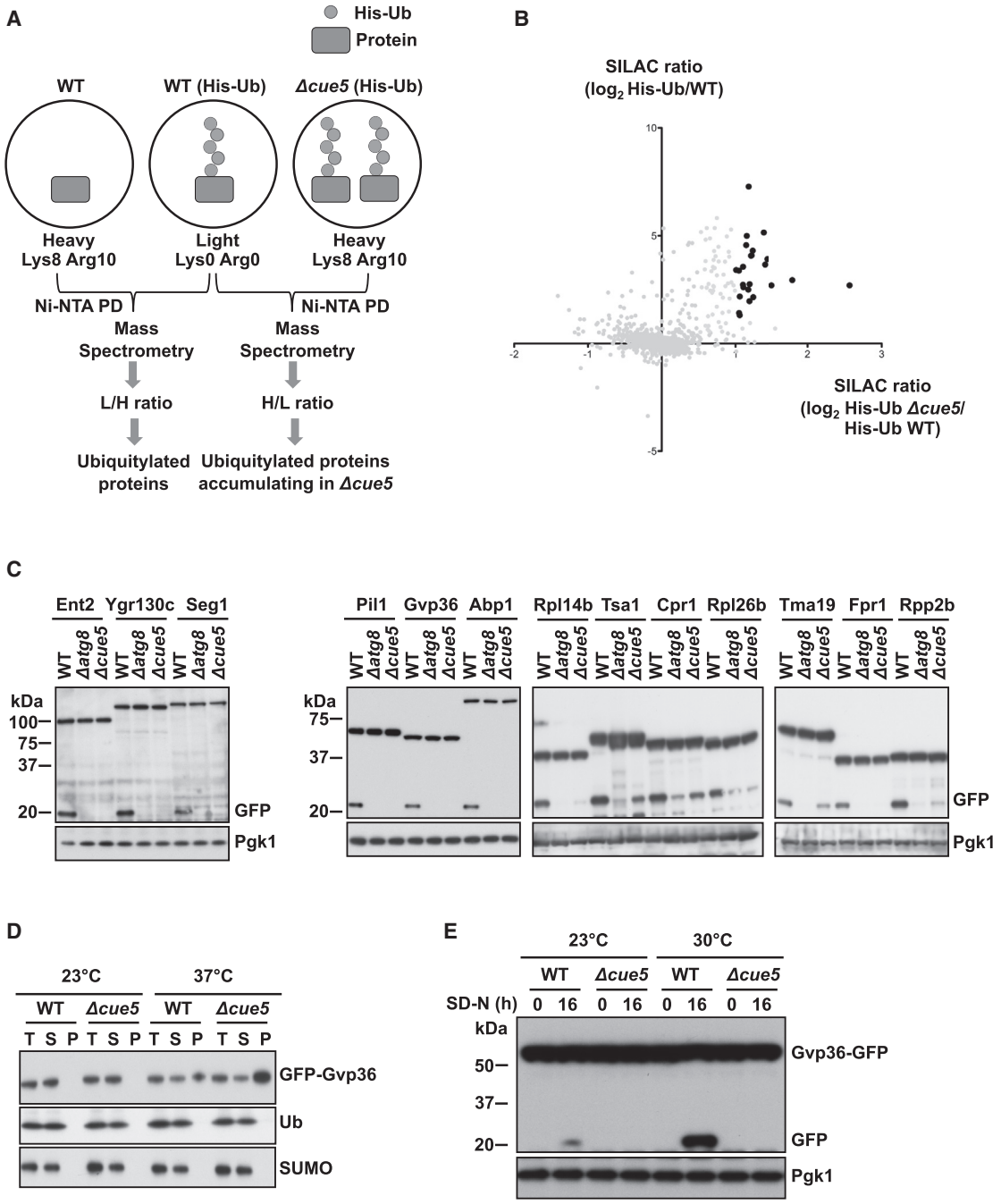


Figure 3. Identification and Analysis of the Cue5-Dependent Autophagic Substrates

(A) Outline of SILAC experiments aimed to identify the ubiquitylated substrates, autophagic degradation of which is dependent on Cue5 upon starvation. Two independent SILAC experiments were conducted. The first was aimed at identifying all ubiquitylated substrates generated in starved cells (His-Ub/WT SILAC ratios measured following Ni-NTA pull-down), and the second was designed to detect ubiquitylated substrates that accumulate upon starvation specifically in the absence of Cue5 (His-Ub *Δcue5*/His-Ub SILAC ratios measured following Ni-NTA pull-down). For detailed description of SILAC mass spectrometry approach, see Experimental Procedures.

(B) Ubiquitylated Cue5-dependent autophagic substrates identified by SILAC mass spectrometry. Two SILAC data sets described in (A) were combined and plotted in a single scatter diagram. Proteins that were found ubiquitylated (y axis; $\log_2$ SILAC ratios above 1) and specifically accumulated further in Cue5-deficient (*Δcue5*) cells (x axis; $\log_2$ SILAC ratios above 1) were considered as high-confidence ubiquitylated substrates of Cue5-dependent autophagic clearance and are shown in black. The list of substrates is presented in Figure S3A, and their validation is in (C).

(C) Validation of the high-confidence Cue5-dependent autophagic substrates identified in (B). C-terminally GFP-tagged proteins were checked for their autophagic degradation by GFP-cleavage assay in WT and Atg8- or Cue5-deficient cells following 16 hr starvation in SD-N medium at 30°C.

(legend continued on next page)

harboring 96 glutamine residues (Htt-96Q) and a version with a shorter polyQ stretch (Htt-20Q), which is much less aggregation prone (Park et al., 2013). When we expressed these constructs in yeast using a copper-inducible promoter, we noticed that Htt-96Q, but not Htt-20Q, affected growth on plates for *Δatg8* cells and, albeit to a lesser extent, also in *Δcue5* and *rsp5-2* mutants (Figure 5A). Thus, Cue5 and Rsp5 confer partial resistance to the cytotoxicity of polyQ proteins in yeast, but additional detoxifying Atg8-dependent pathways (which possibly do not rely on ubiquitylation) appear to exist in yeast as well.

To verify that Cue5 and Rsp5 are involved in the clearance of aggregated Htt-96Q, we performed cell fractionation assays. Whereas only a small fraction of Htt-96Q was found in the pellet fraction of extracts from WT cells, the expanded polyQ protein strongly accumulated in the pellet fractions of mutants defective in Atg8, Cue5, and Rsp5 (Figure 5B), in contrast to its non-aggregation-prone Htt-20Q variant (Figure S5A). Moreover, when WT cells were grown at elevated temperatures (30°C compared to 23°C), not only Htt-96Q accumulated in the pellet fraction, but also a sizable fraction of Atg8 and Cue5 (Figure 5C). This suggests that temperature stress promotes polyQ protein aggregation in yeast cells, which also causes a redistribution of autophagy factors into protein aggregates. We also noticed that Htt-96Q is ubiquitin modified in yeast and that Rsp5 is the critical ubiquitin ligase (Figure S5B). Moreover, immunoprecipitation of both Atg8 and Cue5 (as HA-tagged versions) co-precipitated Htt-96Q (Figure 5D), but not Htt-20Q (Figure S5C). As binding of Htt-96Q to Cue5 depended on the integrity of the CUE domain (Figure 5E), we conclude that Cue5 recognizes ubiquitylated Htt-96Q in yeast. From all of the above findings, we thus conclude that Cue5 is a crucial quality control factor of yeast, which functions by clearing cells from protein aggregates, such as polyQ proteins, by the ubiquitin-linked autophagy pathway.

Human Tollip Is a Functional Homolog of Yeast Cue5

Bioinformatic analysis (DELTA-BLAST) (Boratyn et al., 2012) revealed no obvious human ortholog of Cue5 but identified the CUE-domain-containing Tollip (Toll-interacting protein), a protein previously implicated in innate immunity and endocytosis (Capelluto, 2012; Visvikis et al., 2011), as a candidate. However, Tollip differs from Cue5 by its domain organization (Figure 6A), as it possesses additionally to a CUE domain a large N-terminal extension, harboring a phospholipid-binding C2 domain. On the other hand, Tollip contains two putative AIM (LIR) motifs within this C2 domain (AIM1 and AIM2; Figure 6A), indicating that it seems to possess the structural hallmarks required for a functional ubiquitin-Atg8/LC3 adaptor.

Indeed, by conducting coimmunoprecipitation experiments, we found that Tollip binds the human Atg8 homolog LC3 (Figures 6B and S6A). To test whether the putative AIMs are functional, we generated Tollip variants harboring amino acid alterations in either of the two putative AIM sequences or both (Tollip M1, M2, M1+M2). Coimmunoprecipitation experiments revealed that both motifs contribute to LC3 binding and that Tollip lost this ability if both motifs are defective (Figure 6B). When we tested for ubiquitin-binding activity of Tollip by GST pull-down experiments, we observed that Tollip (as a GST fusion) binds recombinant free His-tagged ubiquitin in vitro (Figure S6B) and also ubiquitin conjugates of HeLa cell extracts (Figure S6C). The interaction of Tollip with ubiquitin conjugates is mediated by its CUE domain, as the CUE-domain mutant variant (Tollip-CUE^{mut} = Tollip-L267A, L268A) is unable to bind ubiquitin conjugates (Figure 6C). Notably, in contrast to a previous report (Mitra et al., 2013), we found no evidence for ubiquitin-binding activity for the C2 domain but solely for the C-terminal CUE-domain-containing region of Tollip (Figures S6B and S6C). Importantly, when we expressed RFP-tagged Tollip in human cells and blocked the fusion of autophagosomes with lysosomes by bafilomycin A1 treatment, Tollip colocalized with ubiquitin and LC3-positive autophagosomes (Figures 6D and S6D), indicating that Tollip is indeed linked to autophagy. Furthermore, a sizable fraction of human Tollip (and LC3, as GFP fusions) is found in the insoluble protein fraction of cells (Figure S6E) analogous to yeast Cue5 and Atg8 (Figure 5C). However, Tollip appears to be recruited to aggregates independent of ubiquitin-conjugate recognition, as the CUE-domain mutant variant (CUE^{mut}) is found in the pellet at the same level as the WT Tollip (Figure S6F).

Strikingly, when expressed in yeast, human Tollip not only bound yeast ubiquitin conjugates and Atg8 in vivo (Figure S6G), but also suppressed the hypersensitivity of yeast Cue5-deficient (*Δcue5*) cells toward Htt-96Q expression (Figure S6H) and restored the autophagic degradation of ubiquitin conjugates in *Δcue5* mutants (Figure S6I). Thus, Tollip fulfills both biochemical and genetic criteria for being a functional human homolog of the *S. cerevisiae* Cue5 despite the differences in their domain arrangements. We therefore conclude that yeast Cue5 and human Tollip define a new class of ubiquitin-Atg8/LC3 adaptors (termed CUET proteins), being also the first found in both lower eukaryotes and vertebrates.

Tollip Clears Human Cells from Huntingtin-Derived PolyQ Proteins

Encouraged by the finding that human Tollip can provide aggregation-prone polyQ protein tolerance in yeast (Figure S6H), we wondered whether Tollip has this capacity in human cells as well. To address this question, we coexpressed an aggregation-prone huntingtin-derived model substrate bearing an expanded polyQ stretch (GFP-Htt-103Q) with Tollip in human HeLa cells. Strikingly, expression of Tollip promoted the degradation of the polyQ protein (Figures 7A and S7A). Importantly, Tollip's activity in polyQ clearance is dose dependent and acts via autophagy and not proteasomal degradation, as indicated by autophagy and proteasome inhibition experiments

(D) Cue5-dependent autophagy substrate Gvp36 accumulates much more in the pellet (P) fraction of Cue5-deficient (*Δcue5*) cells at elevated temperatures compared to WT cells. By contrast, non-aggregation-prone proteins ubiquitin (Ub) and SUMO remain soluble.

(E) C-terminally GFP-tagged Gvp36 was monitored for its autophagic degradation at different temperatures (23°C and 30°C) by GFP-cleavage assay in WT and Cue5-deficient cells.

See also Figure S3.

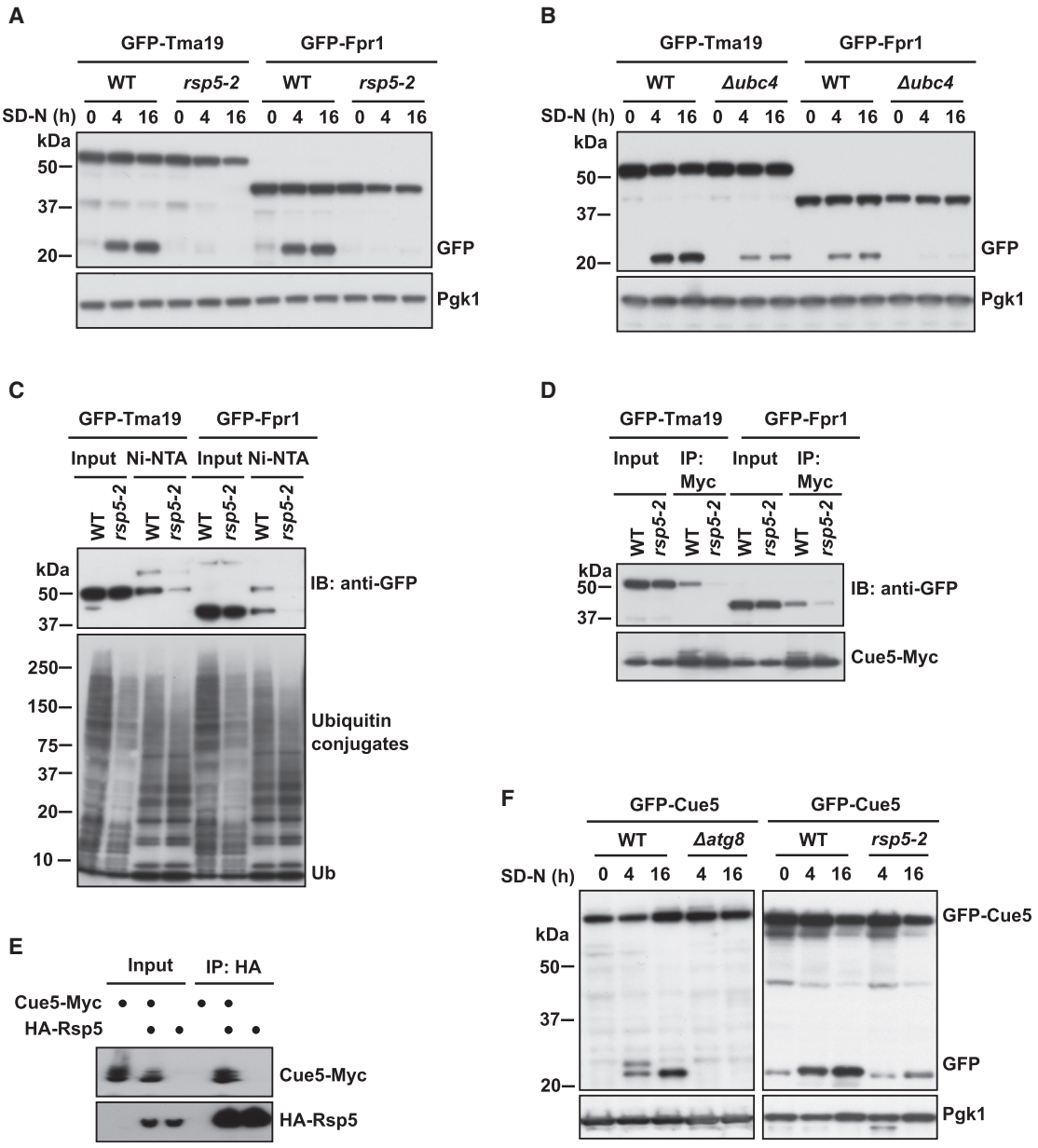


Figure 4. Ubiquitin Ligase Rsp5 Is Linked to Cue5-Dependent Autophagy

(A and B) Two confirmed Cue5-dependent autophagic substrates, Tma19 and Fpr1, require the activity of ubiquitin ligase Rsp5 and ubiquitin-conjugating enzyme Ubc4 for degradation upon starvation. Tma19 and Fpr1 were N-terminally GFP tagged in WT and *rsp5-2* mutant or Ubc4-deficient (*Δubc4*) cells, and their starvation-induced autophagic degradation was analyzed by GFP-cleavage assay.

(C) Ubiquitylation of Tma19 and Fpr1 depends on Rsp5. Denaturing Ni-NTA pull-down was performed to isolate His-tagged ubiquitin conjugates from WT and *rsp5-2* mutant cells expressing GFP-tagged Tma19 and Fpr1.

(D) Interaction of Tma19 and Fpr1 with Cue5 depends on Rsp5. Interaction of GFP-tagged Tma19 and Fpr1 with C-terminally Myc-tagged Cue5 was analyzed in WT and *rsp5-2* mutant cells by coimmunoprecipitation.

(E) Ubiquitin-Atg8 adaptor Cue5 interacts with Rsp5. Interaction of C-terminally Myc-tagged Cue5 with N-terminally HA-tagged Rsp5 was analyzed by coimmunoprecipitation.

(F) Starvation-induced autophagic degradation of Cue5 is dependent on Rsp5. Degradation of N-terminally GFP-tagged Cue5 in WT and Atg8-deficient (*Δatg8*) or *rsp5-2* mutant cells upon starvation was analyzed by GFP-cleavage assay.

See also Figure S4.

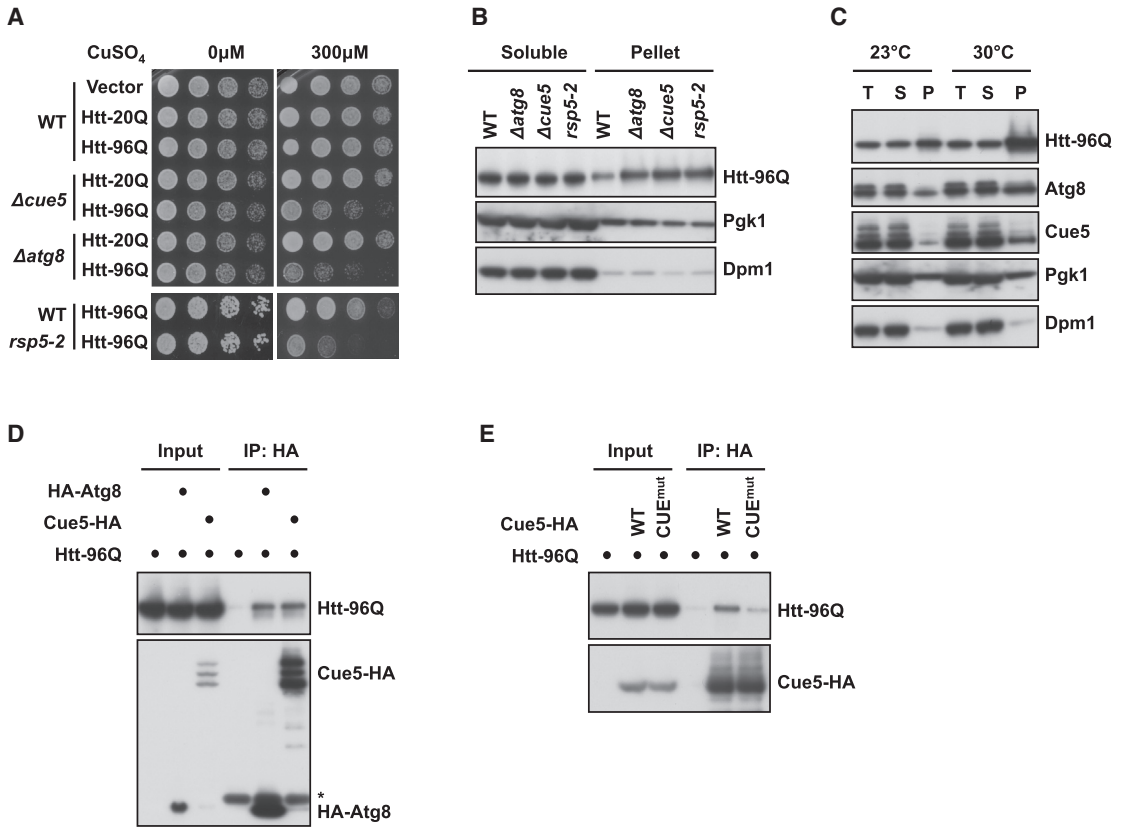


Figure 5. Cue5 and Rsp5 Facilitate the Clearance of Huntingtin-Derived PolyQ Proteins

(A) Toxic effects of expressed model substrates derived from the Huntingtin gene polyQ exon-1 fragment (non-aggregation-prone variant Htt-20Q and highly aggregation-prone variant Htt-96Q) were analyzed in WT, Atg8-, or Cue5-deficient cells and also in *rsp5-2* mutant cells. Htt-20Q and Htt-96Q variants were expressed under *CUP1*-promoter control (300 μM $CuSO_4$).

(B) Clearance of Htt-96Q protein aggregates depends on Atg8, Cue5, and Rsp5. WT, Atg8-, or Cue5-deficient cells and *rsp5-2* mutant cells expressing Htt-96Q variant under *CUP1*-promoter control (100 μM $CuSO_4$) were fractionated and Htt-96Q protein detected in soluble and pellet fractions. Pgk1 and Dpm1 were used as non-aggregation-prone protein controls.

(C) Atg8 and Cue5 accumulate together with Htt-96Q protein aggregates in the pellet (P) fraction at elevated temperatures. Similar to (B) but comparing different temperatures (30°C; 23°C).

(D) Atg8 and Cue5 bind the aggregation-prone protein Htt-96Q. N-terminally HA-tagged Atg8 and C-terminally HA-tagged Cue5 immunoprecipitated Htt-96Q protein expressed under *CUP1*-promoter control (100 μM $CuSO_4$) in coimmunoprecipitation assays. The asterisk denotes the IgG light chain.

(E) Cue5 binding to the aggregation-prone protein Htt-96Q depends on the CUE domain. C-terminally HA-tagged Cue5 WT, but not its CUE-domain mutant variant (CUE^{mut}), immunoprecipitated Htt-96Q protein. Experiment conducted similar to (D).

See also Figure S5.

(Figure 7A). Indeed, when we coexpressed GFP-Htt-103Q either with WT Tollip or its mutant variants defective in LC3 binding (Tollip M1, M2, M1+M2), only WT Tollip promoted the autophagic degradation of polyQ (Figure 7B). Notably, the polyQ protein already partially accumulated in cells when the expressed Tollip protein lacked just one of the two AIMs (Figure 7B). Moreover, autophagic clearance of GFP-Htt-103Q by Tollip was also dependent on its CUE domain, as the CUE-domain mutant variant (CUE^{mut}) failed to promote polyQ degradation (Figure 7C). From these findings, we thus infer that Tollip is highly potent in effectively clearing cells from the overexpressed huntingtin polyQ protein and that both AIMs of Tollip as well as its CUE domain are needed for its full potency. Because overexpression of Tollip does not clear human cells from huntingtin variants harboring shorter polyQ stretches (Htt-20Q; Figure 7D), Tollip appears to target specifically highly aggregation-prone proteins (like Htt-72Q and Htt-103Q) for autophagy (Figure 7D). Remarkably, yeast Cue5 when heterologously expressed in human cells was also able (albeit less potently) to clear cells from expanded polyQ proteins (Htt-103Q) in a dose-dependent manner and requiring functional CUE and AIM binding motifs (Figure 7E). We thus infer that Cue5 and Tollip are functionally similar also in human cells and that this class of ubiquitin-Atg8/LC3 adaptors is particularly potent toward expanded polyQ proteins.

Previous studies have demonstrated that the human ubiquitin-Atg8/LC3 adaptor p62 mediates targeting of polyQ proteins to autophagosomes (Kraft et al., 2010; Pankiv et al., 2007), but whether polyQ proteins are eliminated via p62 from cells has not been directly addressed. To investigate this issue, we first analyzed the potency of p62 and Tollip in autophagic clearance

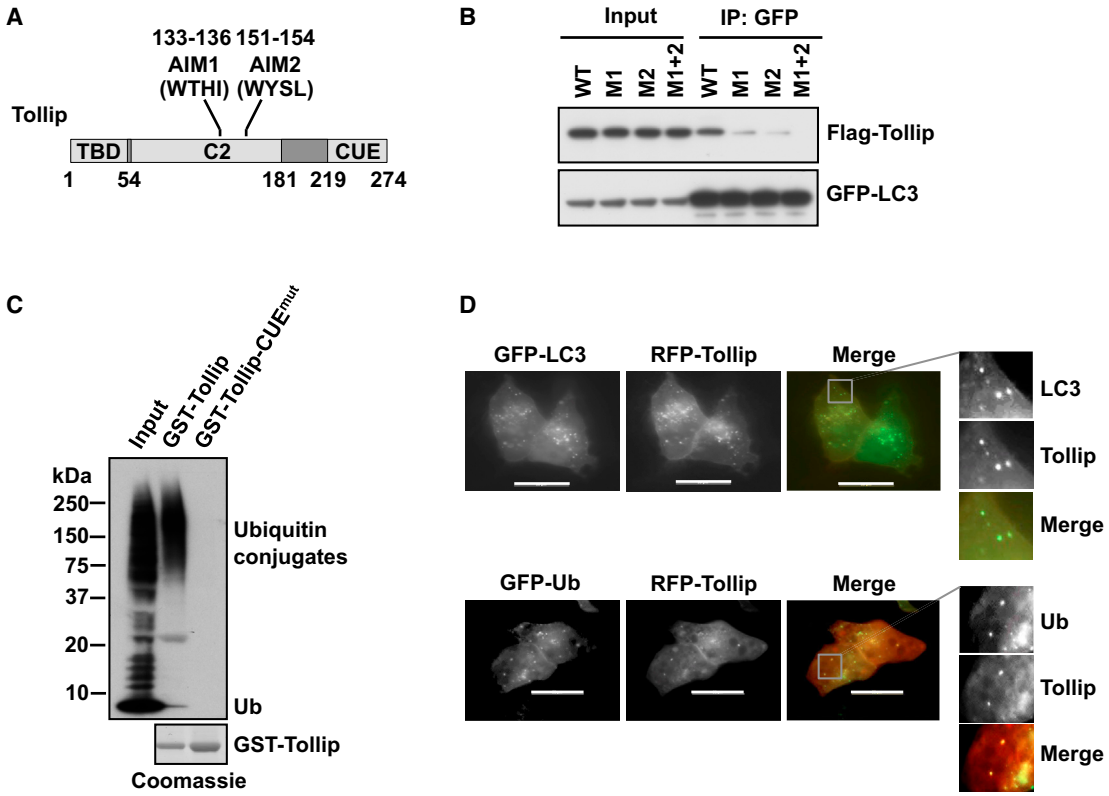


Figure 6. Tollip Is a Human Homolog of Yeast Ubiquitin-Atg8 Adaptor Protein Cue5

(A) Schematic representation of human Tollip protein. Tollip contains a Tom1-binding domain (TBD), a phospholipid-binding C2-domain harboring two putative LC3/Atg8-interacting motifs (AIM1, AIM2), and a ubiquitin-binding CUE domain.

(B) Tollip binding to LC3 is mediated by both of its putative AIMs. N-terminally Flag-tagged WT Tollip or its AIM mutant variants (Tollip-W133A, T134A, H135A, I136A: M1; Tollip-W151A, Y152A, S153A, L154A: M2; Tollip-W133A, T134A, H135A, I136A, W151A, Y152A, S153A, L154A: M1+2), were coexpressed with N-terminally GFP-tagged LC3 in HeLa cells, and their interaction was analyzed by coimmunoprecipitation assays.

(C) Binding of Tollip to ubiquitin conjugates is mediated by its CUE domain. GST pull-down assays were performed using HeLa whole-cell extracts and GST fusions of WT Tollip or its CUE-domain mutant variant (Tollip-CUE^{mut} = Tollip-L267A, L268A).

(D) Tollip colocalizes with LC3 and ubiquitin after blockage of the autophagosome-lysosome fusion in HeLa cells. RFP-Tollip was coexpressed with GFP-LC3 or GFP-ubiquitin (Ub). Following cell treatment with bafilomycin A1 (0.2 μM) for 16 hr, cells were imaged using fluorescence microscopy. Scale bars, 20 μm.

See also Figure S6.

of huntingtin-derived polyQ proteins. In our experimental system (HeLa cells), we found that Tollip was more potent in polyQ protein (Htt-103Q) clearance than p62 (Figure 7F). A possible explanation for this difference came from the finding that Tollip binds endogenous ubiquitin conjugates of human cells apparently tighter than p62, as demonstrated by immunoprecipitation and GST-pull-down experiments (Figures 7G and S7B). Although both adaptors bind free ubiquitin (His ubiquitin) with apparently similar affinities (Figure S7D), Tollip seems to bind purified K48- or K63-linked polyubiquitin chains stronger than p62, in particular those of a longer chain length (Figure S7C).

We further compared Tollip to p62 by depleting the adaptors from human HeLa cells by siRNA (Figures S7E and S7F) and monitored viability of cells expressing either a control (GFP) or the GFP-tagged huntingtin-derived polyQ protein (Htt-103Q). Notably, depletion of Tollip (using either of two different siRNAs) resulted in a higher loss of HeLa cell viability in the face of polyQ protein expression compared to p62 depletion (Figure 7H). Importantly, the general autophagic activity (indicated by the accumulation of lipidated LC3; Klionsky et al., 2012) is not affected when Tollip is depleted (Figure S7G). Because co-depletion of Tollip and p62 resulted in further reduction of cell viability upon expression of huntingtin-derived polyQ protein (Htt-103Q; Figure S7H), the two ubiquitin-Atg8/LC3 adaptors do not seem to function sequentially in a pathway (non-epistatic) but may act cooperatively. In fact, immunoprecipitation of Tollip co-isolated a small fraction of p62 (and NBR1) from cell extracts (Figure S7I), indicating that different adaptors possibly cooperate in some cases by targeting the same cellular aggregate yet perhaps via distinct ubiquitin conjugates and types of ubiquitin modifications. Our findings thus revealed that CUET ubiquitin-Atg8/LC3 adaptors have highly similar functions in yeast and humans and that Tollip is a critical new player in a human safeguarding network that provides protection against the cytotoxicity of polyQ proteins by autophagic clearance.

DISCUSSION

Autophagy, initially appreciated for its role of protein recycling upon cellular starvation, is now seen as a major player for cellular regulation, stem cell maintenance, innate immunity, organelle turnover, and protein quality control. In particular, ubiquitin-dependent autophagy received special attention because it plays a crucial protective role against cytotoxic proteins and aggregates like those linked to several neurodegenerative diseases. Research during the past decades led to the successful identification of the core components of the autophagy pathway. More recently, however, strong focus is put on the discovery of factors that provide substrate specificity, as they act as decision makers in the autophagy pathway (Rogov et al., 2014).

Given that the majority of core components of the autophagy pathway had been initially discovered mainly in yeast (Mizushima et al., 2011), it seemed doubtful that ubiquitin-Atg8 adaptors analogous to p62 are absent in lower eukaryotes. The motivation to search for potential yeast adaptors stemmed not only from the goal of dissecting the selectivity of autophagy in the genetically tractable *S. cerevisiae*, but also from the hope that a potentially identified yeast adaptor might have a hitherto overlooked human homolog.

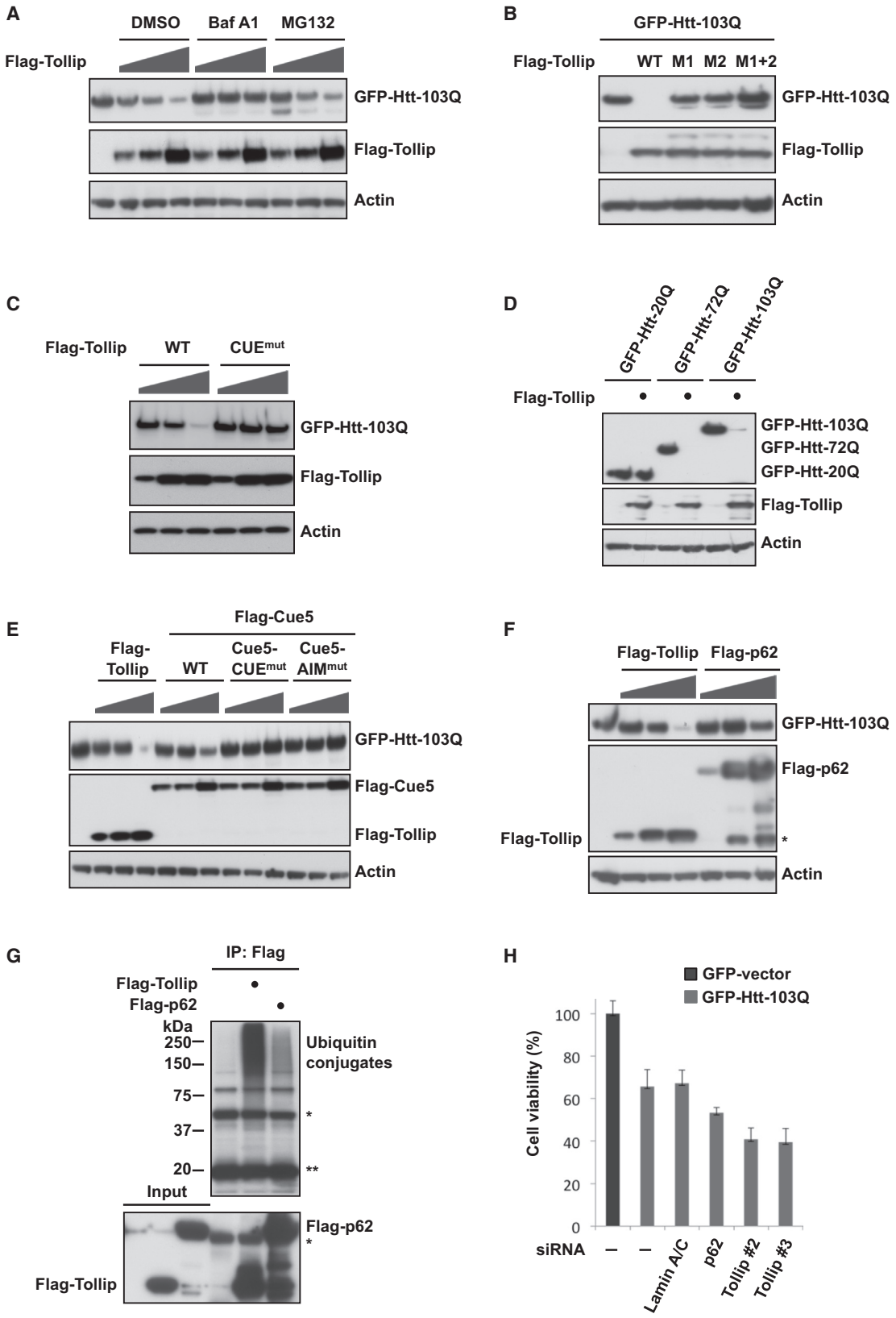
Indeed, the approach that we undertook succeeded in finding a new class of ubiquitin-Atg8/LC3 adaptors coined CUET proteins, which are functionally conserved from yeast to humans. Because previously discovered ubiquitin-Atg8 adaptors are restricted to metazoans (Rogov et al., 2014), CUET proteins might be ancient and perhaps of basic cellular importance. Notably, unlike p62 or NBR1, Cue5 and Tollip do not possess UBA domains, but a CUE domain mediates ubiquitin binding instead. As CUE domains are also found in yeast Cue1 and human GP78 involved in ERAD (Bagola et al., 2013; Chen et al., 2006), Vps9 involved in endocytosis (Donaldson et al., 2003), and yeast Don1 linked to prospore-membrane assembly (Moreno-Borchart et al., 2001), ubiquitin-binding CUE domains seem to be chiefly associated with membrane-linked ubiquitin-dependent processes. Because the CUE domains of Cue5 and Tollip bind monoubiquitin and also polyubiquitin chains of various types, CUET proteins are perhaps tailored for binding heavily and diversely ubiquitylated proteins, such as those found in protein aggregates.

Our findings indicate that CUET proteins act as potent ''guardians of the proteome'' of lower and higher eukaryotes through their ability to clear cells efficiently from protein aggregates by the ubiquitin-dependent autophagy pathway. The discovery that yeast Cue5 collaborates with Ubc4/Ubc5 ubiquitin-conjugating enzymes and the Rsp5 ubiquitin ligase is striking, as these components of the ubiquitin pathway mediate stress tolerance in yeast. Whereas Ubc4 and Ubc5 are heat stress inducible and crucial for proteasome-mediated degradation of abnormal proteins (Seufert and Jentsch, 1990), Rsp5 is required for cell viability under heat-stress conditions (Hoppe et al., 2000). This relationship thus backs the idea of a hierarchically organized protein quality network targeting ubiquitylated abnormal proteins initially to proteasomal degradation; but in the case of formed protein aggregates, proteolytic elimination is achieved by autophagy. Our finding that Cue5 does not detectably bind a short-lived ubiquitin-proteasome pathway substrate suggests that additional parameters such as protein aggregation may be relevant for cargo selection by Cue5 as well.

The identification of Tollip as a human ubiquitin-Atg8/LC3 adaptor and highly potent mediator of autophagy-linked elimination of polyQ proteins like huntingtin has numerous implications. Notably, Tollip has been reported previously to copurify with cellular polyQ aggregates (Doi et al., 2004), and it was also found to be downregulated in brain tissue samples from aged and Alzheimer's-disease-affected humans (Cribbs et al., 2012), emphasizing its potential disease-related role. Moreover, it has been suggested that Tollip mediates polyQ protein tolerance through its ability to stimulate their aggregation (Oguro et al., 2011). However, our findings revealed that Tollip, in addition to this proposed tolerance activity, directly mediates polyQ protein elimination via the autophagy pathway. Indeed, Tollip's potency in autophagic clearance is striking, as overexpressed huntingtin-derived expanded polyQ proteins became virtually undetectable when Tollip was overexpressed as well (Figures 7A and S7A).

Interestingly, Tollip is substantially more potent in polyQ protein clearance than p62 in HeLa cells (Figure 7F). Furthermore, expression of polyQ proteins is more cytotoxic in HeLa cells deficient in Tollip compared to cells depleted in p62 (Figures 7H and S7H). Although the relative potency might be different in other cell types, Tollip appears to bind ubiquitin conjugates—in particular, polyubiquitylated proteins—better than p62, both in vitro and in vivo (Figures 7G, S7B, and S7C). Indeed, the expression of different ubiquitin-Atg8/LC3 adaptors with distinct binding properties and ubiquitin-modification affinities might be advantageous for cells, as this may allow recognition of protein aggregates that are diversely ubiquitin modified by different ubiquitin ligases. Evidence for this idea comes from our findings that a small fraction of p62 (and NBR1) copurifies with Tollip in cells (Figure S7I) and that polyQ protein expression is even more toxic when both p62 and Tollip are depleted, compared to situations in which only one adaptor is absent (Figure S7H). We infer from these findings that Tollip and p62 in fact sometimes cooperate in autophagy by targeting the same cellular aggregate. It also seems reasonable to assume that the multiplicity in ubiquitin-Atg8/LC3 adaptors found in metazoans might have evolved to satisfy the increasing needs of more complex cells to cope with protein aggregation.

Although yeast Cue5 and human Tollip are functionally homologous ubiquitin-Atg8/LC3 adaptors, the two proteins also differ. One difference is the relative positioning of CUE and AIM sequences in Cue5 and Tollip. However, this seems inconsequential because the proteins function as bridging adaptors, connecting ubiquitylated substrates/aggregates to Atg8/LC3 and autophagosomes. A more notable difference between the two CUET proteins is the additional presence of a Tom1-binding domain (TBD) and a phospholipid-binding C2 domain within Tollip's N-terminal extension (Figure 6A). However, these additional domains are potentially linked to Tollip's extra functions. In addition to its role in autophagy described here, Tollip participates in Toll-like receptor (TLR)-mediated innate immunity responses and protein traffic by the endocytic pathway (Capelluto, 2012). Whereas CUE-mediated ubiquitin-binding and C2-domain-mediated phospholipid-binding seems crucial for most of



(legend on next page)

Tollip's proposed functions in cell signaling and membrane trafficking (Capelluto, 2012), binding of Tollip to Tom1, an ESCRT-0 component, mediates, e.g., the recruitment of clathrin to endosomes (Katoh et al., 2006). Our finding that the two AIM elements are present directly within Tollip's C2 domain is at first sight surprising; on the other hand, it suggests that this arrangement might perhaps function as a switch, i.e., that LC3 binding to the C2 domain of Tollip might channel the activity of this otherwise multifunctional protein toward autophagy. Indeed, a conserved residue (His135) in the C2 domain critically required for Tollip's binding to phosphoinositides (Ankem et al., 2011) lies precisely within the putative AIM (AIM1) important for interaction with LC3 (Figures 6A and 6B). At any rate, our findings highlight the Swiss-Army-knife-like property of Tollip, as the protein, in addition to its role as multitasking adaptor employed in various plasma membrane-to-cytosol signaling and endocytic protein trafficking pathways, also functions as ubiquitin-Atg8/LC3 adaptor in the autophagy pathway. We propose that this feature could lead to situations in which aggregation-prone polyQ proteins like huntingtin perhaps contribute to cytotoxicity and neurodegenerative diseases by interfering with other functions of the adaptor.

EXPERIMENTAL PROCEDURES

Yeast Strains and Constructs

Yeast (*S. cerevisiae*) strains and constructs used in this study are listed in Tables S1 and S2, respectively.

Yeast Starvation, GFP Cleavage, and Cell Fractionation Assays

Yeast cells were first cultured in YPD media to the log phase and were then switched to synthetic minimal medium lacking nitrogen (SD-N) for indicated times. For GFP-cleavage assays, autophagy substrates are GFP tagged (e.g., GFP-Atg8), and their vacuolar degradation upon starvation is monitored by the accumulation of the released GFP moiety, which is highly stable and escapes autophagic degradation. For cell fractionation assays, yeast total cell extracts were prepared by cell disruption using bead-beater, precleared at 2,000 × *g* and fractionated at 16,000 × *g* for 10 min to separate proteins into soluble and insoluble pellet fraction. Detailed descriptions are provided in the Extended Experimental Procedures.

Biochemical and Molecular Biology Techniques

The biochemical and molecular biology techniques used in this study are standard procedures. Detailed descriptions for individual methods are provided in the Extended Experimental Procedures.

SILAC Mass Spectrometry

To identify ubiquitylated substrates targeted for autophagic degradation by Cue5 upon starvation, a SILAC-based mass spectrometry protocol was used. WT or *Δcue5* yeast cells deficient in biosynthesis of lysine and arginine (*Δlys1 Δarg4*) expressing His-tagged ubiquitin (His-Ub) were grown for at least ten divisions in synthetic complete (SC) media supplemented either with unlabeled (Lys0 and Arg0; light) or heavy isotope-labeled amino acids (Lys8 and Arg10; heavy) from Cambridge Isotope Laboratories. Log-phase *Δcue5* cells cultured in heavy media and WT cells grown in light media were then subjected to nitrogen starvation in SD-N media for 16 hr, harvested, and combined together at equal amounts. His-Ub conjugates were then isolated using denaturing Ni-NTA pull-down and were separated on 4%–12% Bis-Tris gel. The whole lane was excised in ten slices, and proteins were digested with trypsin and analyzed by LC-MS/MS.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.05.048.

ACKNOWLEDGMENTS

We thank F. den Brave, F. Paasch, and F. Wilfling for comments on the manuscript; U. Hartl and E. Lemichez for materials; C. Boulegue for mass spectrometry analysis; B. Habermann for help with bioinformatic analysis; T. Ammon for help with mammalian cell culture techniques; and A. Strasser for technical assistance. S.J. is supported by Max Planck Society, Deutsche Forschungsgemeinschaft, Center for Integrated Protein Science Munich, ERC Advanced Grant, and the Louis-Jeantet Foundation.

Figure 7. Tollip Clears Human Cells from Huntingtin-Derived PolyQ Proteins

(A) Tollip promotes the autophagic clearance of aggregation-prone huntingtin-derived polyQ proteins from human cells. N-terminally Flag-tagged Tollip and GFP-tagged aggregation prone Htt-103Q variant were co-overexpressed in HeLa cells, and Htt-103Q protein levels were analyzed after treatment with DMSO, autophagy inhibitor bafilomycin A1 (Baf A1, 0.2 μM), or proteasome inhibitor MG132 (10 μM) for 16 hr.

(B) Tollip promotes the clearance of aggregation-prone huntingtin-derived polyQ proteins from human cells depending on interaction with LC3. N-terminally Flag-tagged WT Tollip protein or its AIM mutant variants (M1, M2, M1+2) were co-overexpressed with N-terminally GFP-tagged Htt-103Q in HeLa cells, and Htt-103Q protein levels were analyzed.

(C) Tollip promotes the clearance of aggregation-prone huntingtin-derived polyQ proteins from human cells depending on its CUE domain. N-terminally Flag-tagged WT Tollip protein or its CUE-domain mutant variant (CUE^{mut}) were co-overexpressed with N-terminally GFP-tagged Htt-103Q in HeLa cells, and Htt-103Q protein levels were analyzed.

(D) Tollip specifically promotes the degradation of highly aggregation-prone huntingtin-derived polyQ proteins. Overexpressed Htt-72Q and Htt-103Q variants with expanded polyQ stretches, but not the shorter Htt-20Q variant, are cleared by Tollip overexpression. Experiment conducted similar to (A).

(E) Yeast Cue5 expressed in human cells promotes the clearance of aggregation-prone Htt-103Q protein depending on CUE domain and AIM. N-terminally Flag-tagged Tollip and Cue5 WT or its CUE-domain mutant (Cue5-CUE^{mut}) and AIM mutant (Cue5-AIM^{mut}) variants were co-overexpressed with GFP-Htt-103Q in HeLa cells and Htt-103Q protein levels were analyzed.

(F) Tollip is more potent in clearance of the aggregation-prone Htt-103Q protein than p62. N-terminally Flag-tagged Tollip or p62 were co-overexpressed with GFP-Htt-103Q in HeLa cells, and Htt-103Q protein levels were analyzed. The asterisk denotes the degradation products of Flag-p62.

(G) Tollip binds endogenous ubiquitin conjugates of human cells better than p62. N-terminally Flag-tagged Tollip or p62 overexpressed in HeLa cells were immunoprecipitated from whole-cell extracts with anti-Flag affinity matrix. Coimmunoprecipitated ubiquitin conjugates were analyzed by immunoblotting using anti-ubiquitin antibody (P4D1). The asterisks denote the light and heavy chains of IgG.

(H) Depletion of Tollip results in a higher loss of HeLa cell viability upon overexpression of aggregation-prone Htt-103Q protein compared to p62 depletion. Tollip and p62 were depleted by specific siRNAs in HeLa cells overexpressing GFP-Htt-103Q, and cell viability was checked by MTT assay after 48 hr. siRNA against Lamin A/C was used as a control. The results are the average of three independent studies, and error bars represent SD.

See also Figure S7.

Received: February 8, 2014
Revised: April 29, 2014
Accepted: May 19, 2014
Published: July 17, 2014

REFERENCES

Ankem, G., Mitra, S., Sun, F.R., Moreno, A.C., Chutvirasakul, B., Azurmendi, H.F., Li, L.W., and Capelluto, D.G.S. (2011). The C2 domain of Tollip, a Toll-like receptor signalling regulator, exhibits broad preference for phosphoinositides. Biochem. J. *435*, 597–608.

Bachmair, A., Finley, D., and Varshavsky, A. (1986). In vivo half-life of a protein is a function of its amino-terminal residue. Science *234*, 179–186.

Bagola, K., von Delbrück, M., Dittmar, G., Scheffner, M., Ziv, I., Glickman, M.H., Ciechanover, A., and Sommer, T. (2013). Ubiquitin binding by a CUE domain regulates ubiquitin chain formation by ERAD E3 ligases. Mol. Cell *50*, 528–539.

Bence, N.F., Sampat, R.M., and Kopito, R.R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. Science *292*, 1552–1555.

Boratyn, G.M., Schäffer, A.A., Agarwala, R., Altschul, S.F., Lipman, D.J., and Madden, T.L. (2012). Domain enhanced lookup time accelerated BLAST. Biol. Direct *7*, 12.

Capelluto, D.G.S. (2012). Tollip: a multitasking protein in innate immunity and protein trafficking. Microbes Infect. *14*, 140–147.

Chen, B., Mariano, J., Tsai, Y.C., Chan, A.H., Cohen, M., and Weissman, A.M. (2006). The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. Proc. Natl. Acad. Sci. USA *103*, 341–346.

Choi, A.M.K., Ryter, S.W., and Levine, B. (2013). Autophagy in human health and disease. N. Engl. J. Med. *368*, 651–662.

Cribbs, D.H., Berchtold, N.C., Perreau, V., Coleman, P.D., Rogers, J., Tenner, A.J., and Cotman, C.W. (2012). Extensive innate immune gene activation accompanies brain aging, increasing vulnerability to cognitive decline and neurodegeneration: a microarray study. J. Neuroinflammation *9*, 179.

Doi, H., Mitsui, K., Kurosawa, M., Machida, Y., Kuroiwa, Y., and Nukina, N. (2004). Identification of ubiquitin-interacting proteins in purified polyglutamine aggregates. FEBS Lett. *571*, 171–176.

Donaldson, K.M., Yin, H.W., Gekakis, N., Supek, F., and Joazeiro, C.A.P. (2003). Ubiquitin signals protein trafficking via interaction with a novel ubiquitin binding domain in the membrane fusion regulator, Vps9p. Curr. Biol. *13*, 258–262.

Fang, N.N., Ng, A.H., Measday, V., and Mayor, T. (2011). Hul5 HECT ubiquitin ligase plays a major role in the ubiquitylation and turnover of cytosolic misfolded proteins. Nat. Cell Biol. *13*, 1344–1352.

Feng, Y., He, D., Yao, Z., and Klionsky, D.J. (2014). The machinery of macroautophagy. Cell Res. *24*, 24–41.

Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu. Rev. Biochem. *78*, 477–513.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature *441*, 885–889.

Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H.D., and Jentsch, S. (2000). Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. Cell *102*, 577–586.

Kaganovich, D., Kopito, R., and Frydman, J. (2008). Misfolded proteins partition between two distinct quality control compartments. Nature *454*, 1088–1095.

Kang, R.S., Daniels, C.M., Francis, S.A., Shih, S.C., Salerno, W.J., Hicke, L., and Radhakrishnan, I. (2003). Solution structure of a CUE-ubiquitin complex reveals a conserved mode of ubiquitin binding. Cell *113*, 621–630.

Katoh, Y., Imakagura, H., Futatsumori, M., and Nakayama, K. (2006). Recruitment of clathrin onto endosomes by the Tom1-Tollip complex. Biochem. Biophys. Res. Commun. *341*, 143–149.

Kim, Y.E., Hipp, M.S., Bracher, A., Hayer-Hartl, M., and Hartl, F.U. (2013). Molecular chaperone functions in protein folding and proteostasis. Annu. Rev. Biochem. *82*, 323–355.

Kirkin, V., Lamark, T., Sou, Y.S., Bjørkøy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P., et al. (2009a). A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol. Cell *33*, 505–516.

Kirkin, V., McEwan, D.G., Novak, I., and Dikic, I. (2009b). A role for ubiquitin in selective autophagy. Mol. Cell *34*, 259–269.

Klionsky, D.J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Nat. Rev. Mol. Cell Biol. *8*, 931–937.

Klionsky, D.J., Abdalla, F.C., Abeliovich, H., Abraham, R.T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J.A., et al. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy *8*, 445–544.

Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., Ueno, T., Hara, T., Mizushima, N., Iwata, J., Ezaki, J., Murata, S., et al. (2007). Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell *131*, 1149–1163.

Kraft, C., Peter, M., and Hofmann, K. (2010). Selective autophagy: ubiquitin-mediated recognition and beyond. Nat. Cell Biol. *12*, 836–841.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27–42.

Levine, B., Mizushima, N., and Virgin, H.W. (2011). Autophagy in immunity and inflammation. Nature *469*, 323–335.

Mann, M. (2006). Functional and quantitative proteomics using SILAC. Nat. Rev. Mol. Cell Biol. *7*, 952–958.

Mitra, S., Traughber, C.A., Brannon, M.K., Gomez, S., and Capelluto, D.G. (2013). Ubiquitin interacts with the Tollip C2 and CUE domains and inhibits binding of Tollip to phosphoinositides. J. Biol. Chem. *288*, 25780–25791.

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. Annu. Rev. Cell Dev. Biol. *27*, 107–132.

Moreno-Borchart, A.C., Strasser, K., Finkbeiner, M.G., Shevchenko, A., Shevchenko, A., and Knop, M. (2001). Prospore membrane formation linked to the leading edge protein (LEP) coat assembly. EMBO J. *20*, 6946–6957.

Noda, T., and Ohsumi, Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem. *273*, 3963–3966.

Oguro, A., Kubota, H., Shimizu, M., Ishiura, S., and Atomi, Y. (2011). Protective role of the ubiquitin binding protein Tollip against the toxicity of polyglutamine-expansion proteins. Neurosci. Lett. *503*, 234–239.

Ohsumi, Y. (2001). Molecular dissection of autophagy: two ubiquitin-like systems. Nat. Rev. Mol. Cell Biol. *2*, 211–216.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. *282*, 24131–24145.

Park, S.H., Kukushkin, Y., Gupta, R., Chen, T., Konagai, A., Hipp, M.S., Hayer-Hartl, M., and Hartl, F.U. (2013). PolyQ proteins interfere with nuclear degradation of cytosolic proteins by sequestering the Sis1p chaperone. Cell *154*, 134–145.

Piwko, W., and Jentsch, S. (2006). Proteasome-mediated protein processing by bidirectional degradation initiated from an internal site. Nat. Struct. Mol. Biol. *13*, 691–697.

Pohl, C., and Jentsch, S. (2009). Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. Nat. Cell Biol. *11*, 65–70.

Prag, G., Misra, S., Jones, E.A., Ghirlando, R., Davies, B.A., Horazdovsky, B.F., and Hurley, J.H. (2003). Mechanism of ubiquitin recognition by the CUE domain of Vps9p. Cell *113*, 609–620.

Ravid, T., and Hochstrasser, M. (2008). Diversity of degradation signals in the ubiquitin-proteasome system. Nat. Rev. Mol. Cell Biol. *9*, 679–690.

Rogov, V., Dötsch, V., Johansen, T., and Kirkin, V. (2014). Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. Mol. Cell *53*, 167–178.

Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *10*, 398–409.

Seufert, W., and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. EMBO J. *9*, 543–550.

Shih, S.C., Prag, G., Francis, S.A., Sutanto, M.A., Hurley, J.H., and Hicke, L. (2003). A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. EMBO J. *22*, 1273–1281.

Stefani, M., and Dobson, C.M. (2003). Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J. Mol. Med. *81*, 678–699.

Visvikis, O., Boyer, L., Torrino, S., Doye, A., Lemonnier, M., Lorès, P., Rolando, M., Flatau, G., Mettouchi, A., Bouvard, D., et al. (2011). Escherichia coli producing CNF1 toxin hijacks Tollip to trigger Rac1-dependent cell invasion. Traffic *12*, 579–590.