Characterization of CHIP, a ubiquitin ligase involved in neurodegenerative disease

Senior Honors Thesis

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Abstract

Many neurodegenerative diseases are characterized by protein misfolding and aggregation. The frontline in defending against such insults is the cell's protein quality control system, which includes both chaperones as well as the ubiquitin proteasome system (UPS). The latter targets proteins for proteasomal degradation via the covalent attachment of the small peptide ubiquitin. Here, we characterize the brain-expressed ubiquitin ligase called CHIP (C-terminus of Hsc70-interacting protein) that, along with other components of the UPS, has been implicated in neurodegenerative diseases.

We describe CHIP's ability to interact productively with a group of ubiquitin conjugating enzymes (E2 enzymes) with which it is predicted to associate. Furthermore, we present a situation in which one E2 attaches a single ubiquitin to substrate, and another extends the ubiquitin chain, thus exhibiting greater polyubiquitination activity when combined than each E2 on its own.

Additionally, the E2 enzyme Ube2w is shown to ubiquitinate CHIP itself with particularly great efficiency. We find that proteins containing ubiquitin interacting motifs (UIMs) bind preferentially to ubiquitinated, rather than unmodified, CHIP. The recruitment of UIM-containing proteins such as the deubiquitinating enzyme ataxin-3 is significant to ubiquitination reactions, as they contribute to editing the type of ubiquitin chains that are formed. As such, they are necessary to forming ubiquitin chains of the proper linkage type and length in order to successfully target misfolded protein substrates for degradation.

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Figure 1: Data obtained by Eszter Zavodszky and Matthew Scaglione, and figure compiled by Matthew Scaglione.

Figure 2: Data obtained by Eszter Zavodszky. Part (A) formatted with assistance from Matthew Scaglione, part (B) made by Eszter Zavodszky.

Figure 3: Data obtained by Eszter Zavodszky, and figure compiled jointly with Matthew Scaglione.

Figure 4: Data obtained by Eszter Zavodszky. Part (A) formatted by Matthew Scaglione, Parts (B), (C), and (D) formatted by Eszter Zavodszky.

Figure 5: Experiment as well as S5a and CHIP blots done by Eszter Zavodszky. Ataxin-3 blot done by Matthew Scaglione. Figure formatted by Eszter Zavodszky.

Figure 6: Data and figures borrowed with permission from Matthew Scaglione.

Figure 7: Data and figure by Eszter Zavodszky.

Figure 8: Cartoon created by Eszter Zavodszky.

Introduction

Neurodegenerative diseases are a devastating and at present, unavoidable, companion of aging for many adults. A hallmark of most of these diseases, including Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis, and Huntington's Disease, is protein misfolding and consequent aggregation. Within this broad class of proteinopathies, polyglutamine expansion diseases such as Huntington's disease, several spinocerebellar ataxias, spinobulbar muscular atrophy, and dentatorubral-pallidoluysian atrophy form a distinct subclass. These diseases are marked by an expanded CAG repeat in the gene that results in an abnormally long glutamine tract in the affected protein. Although the affected proteins are unrelated, pathogenesis is thought to occur by a similar mechanism (Williams & Paulson, 2008). Like many other neurodegenerative diseases mentioned above, individuals affected by polyglutamine disorders exhibit intraneuronal inclusions of aggregated protein. Though this was thought to be the disease-causing agent for quite some time, current research suggests that inclusions might actually be protective, sequestering the misfolded disease protein (Williams & Paulson, 2008). Instead, neuronal toxicity might be a result of the misfolded protein engaging in abnormal interactions and forming protein complexes with either altered or novel functions (Williams & Paulson, 2008). Additionally, the misfolded protein likely forms toxic oligomers that have the potential to disrupt the stability of other proteins – for example, disrupting macromolecular complexes (Williams & Paulson, 2008). Little is known about why neurons are affected to such a great extent by alterations to protein homeostasis – it is, however, possible that they are particularly sensitive to even subtle alterations in macromolecular complexes (Williams & Paulson, 2008).

The literature shows that the toxicity of polyglutamine species is often modulated by components of the chaperone and protein degradation systems (Williams & Paulson, 2008). Understanding how these systems function and are regulated consequently might lead to the development of therapeutic strategies based on protein quality control. The protein quality control system includes the chaperone system, the purpose of which is to fold newly created proteins, as well as to attempt to refold misfolded proteins. This action is critical, as the exposed hydrophobic residues present on unfolded proteins consequently promote their aggregation and lead, for example, to the formation of the aforementioned toxic oligomeric species (Cyr et al., 2002). Such exposed hydrophobic residues are bound by chaperones, which prevent aggregation and assist in folding (Cyr et al., 2002; Wickner et al., 1999). Chaperones include both constitutively expressed proteins (the heat shock cognate, or HSC family), and inducible chaperones (the heat shock protein, or HSP family), but their functions greatly overlap (Dickey et al., 2007). Some studies suggest that chaperone activity declines with aging, as does the activity of heat shock factor 1 (HSF1), the transcription factor responsible for inducing the HSP chaperones (Dickey et al., 2007). Reduced chaperone and HSF1 activity contributes to the accumulation or proteins, such as that in neurodegenerative diseases which are in turn associated with aging (Dickey et al., 2007).

The other major component of cellular protein quality control – and the focus of this work – is the ubiquitin-proteasome system (UPS). When the chaperone system is unable to refold misfolded proteins, these proteins are instead targeted for degradation by the 26S proteasome (Murata et al., 2001). The proteasome is essentially the eukaryotic "garbage disposal" for proteins, consisting of a 20S central chamber with proteolytic active sites and two flanking 19S regulatory subunits that each contain a ring of six ATPases (Wickner et al., 1999).

In the UPS pathway, a small protein called ubiquitin is attached to protein substrates by the

concerted action of three proteins: a ubiquitin activating enzyme (E1), a ubiquitin conjugating

enzyme (E2)[1], and a ubiquitin ligase (E3) (Pickart, 2001; Murata et al., 2003; Xu et al., 2008;

Ardley & Robinson, 2004). A thioester bond is formed between the C-terminal glycine of

ubiquitin and a cysteine residue in the active site of the E1, which is then transferred to a

cysteine in the active site of the E2 (Cyr et al., 2002). Next, the E3 functions via one of two

mechanisms to mediate the transfer of ubiquitin to the substrate: HECT (homologous to E6-

associated protein-C-terminus) type ligases contain an active site cysteine themselves, which

accepts ubiquitin before eventually transferring it to the substrate (Cyr et al., 2002). On the other

hand, members of the RING/U-box (really interesting new gene) family of ligases do not contain

an active site cysteine, and instead mediate ubiquitination by bringing the E2 and substrate into

close proximity (Cyr et al., 2002). As a result, an isopeptide bond is formed between the C-

terminal glycine residue of ubiquitin and the ε-amino group of a lysine on the substrate protein

(Pickart, 2001; Xu et al., 2008). Often, more than one ubiquitin is attached one after the other,

forming a polyubiquitin chain. As ubiquitin itself has multiple lysines, the types of linkages

present in the chains themselves have important effects on the fate of the substrate. For example,

K48- linked polyubiquitin chains are generally targeted to the proteasome for degradation

(Pickart, 2001; Xu et al., 2008; Murata et al., 2003) whereas K63-linked chains are associated

with protein trafficking (Xu et al., 2008) and autophagic clearance of protein inclusions (Tan et

al., 2007).

It is important to note the hierarchical nature of the enzymes involved in ubiquitination:

in a given organism, there are only a couple of E1 enzymes (in some cases, just one), a few

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[1] The literature is quite inconsistent regarding the naming conventions employed for ubiquitin conjugating enzymes (E2s). While some researchers employ the "Ube2_" system, most E2s have an alternate name, often in the "Ubc_" or "UbcH_" format. Whenever possible, multiple names of E2s will be clarified.

dozen E2s, and hundreds of E3s (Pickart, 2001; Xu et al., 2008). As such, we come upon the problem of substrate specificity. While some (Pickart, 2001) refer to the E3 as the primary determinant of specificity of ubiquitination, others (Xu et al., 2008, Christensen et al., 2007) explain that the particular combination of E2 and E3 enzymes affect the type of ubiquitination and the fate of the ubiquitinated substrates.

Overall, the research that follows seeks to characterize an important, brain-expressed ubiquitin ligase called CHIP (C-terminus of Hsc70-interacting protein). CHIP sits at the intersection of the chaperone pathway and the UPS. As such, it is a key player in protein triage: the identification and sorting of misfolded proteins to the chaperone or proteasomal pathways (Dickey et al., 2007). One of its notable features is a tetratricopeptide repeat (TPR), through which it associates with the carboxyl-terminus of the chaperones Hsp70, Hsc70, and Hsp90 (Murata et al., 2003). All of these chaperones have been implicated in various neurodegenerative conditions, including Huntington's Disease, Parkinson's Disease, and Alzheimer's Disease (Dickey et al., 2007). It is through interaction with the aforementioned chaperones that CHIP is able to act as a bridge between protein refolding and degradation: it preferentially ubiquitinates misfolded substrates that have been captured by the chaperone, and does not function to ubiquitinate free misfolded protein (Murata et al., 2001). Additionally, it is worth mention that once misfolded proteins have been depleted, CHIP regulates stress recovery by ubiquitinating Hsp70 and targeting it for degradation, thus returning to basal levels (Qian et al., 2006)

Another critical region of CHIP is its U-box domain, which resembles a RING domain (Murata et al., 2003), but differs in that it does not bind zinc (Cyr et al., 2002). Nevertheless, CHIP is functionally similar to zinc-containing RING-type E3 ligases (Cyr et al., 2002), thus

earning it a place as a member of the RING/U-box superfamily (Xu et al., 2008). The U-box

domain is necessary for CHIP to function as a ubiquitin ligase, as it allows for interactions with

E2 enzymes (Cyr et al., 2002). Deletion mutants of this region have no ubiquitination activity

(Murata et al., 2001).

CHIP and other components of the UPS have been implicated in neurodegenerative

diseases. The UPS is recruited to protein inclusions that are characteristic of many such diseases

(Murata et al., 2003). Additionally, the failure (either the malfunction or overload) of the protein

quality control system is thought to be the cause of several neurodegenerative diseases (Murata

et al., 2003; Ardley & Robinson, 2004). It is then perhaps unsurprising that overexpression of

CHIP reduced the amount of the mutant protein species and ameliorated the disease phenotype in

mice with spinal and bulbar muscular atrophy, a polyglutamine expansion disease (Adachi et al.,

2007). Similar results were obtained by other researchers both *in vitro* and in a zebrafish

polyglutamine disease model (Miller et al., 2005). CHIP was also found to be protective against

ataxin-1 aggregation, and was shown to suppress eye degeneration in a fly model of

Spinocerebellar Ataxia Type 1 (Al-Ramahi et al., 2006). The beneficial effects of CHIP

overexpression extend beyond the polyglutamine disease family: in cell culture, CHIP was found

to enhance the degradation of mutant superoxide dismutase, the disease protein in familial

amyotrophic lateral sclerosis (Choi et al., 2004). Similarly, CHIP colocalizes to Lewy bodies

with α-synuclein (found in Parkinson's disease), and enhances its degradation via both

proteasomal and lysosomal pathways (Shin et al., 2005). On the other hand, Huntington's

Disease mice with reduced levels of CHIP show a worsening of the disease phenotype as well as

decreased longevity (Miller et al., 2005) Mice with Spinocerebellar Ataxia Type 3 (SCA3) also

demonstrate accelerated disease progression on a CHIP haploinsufficient background (Williams

et al., 2009). Furthermore, these mice exhibit more soluble microaggregates of the SCA3 disease protein ataxin-3 (Williams et al., 2009), a correlation that supports the aforementioned model of toxicity in polyglutamine diseases. Again, CHIP seems to be relevant beyond polyglutamine disease: mice lacking CHIP showed increased tau aggregation – a component of neurofibrillary tangles in Alzheimer's Disease (Sahara et al., 2005). Overall, the similar results found throughout the literature in a variety of disease models highlight the significance of the protein quality control system, particularly the ubiquitin ligase CHIP. Although one study claims that protein context is an important determinant of CHIP's role in modulating aggregation (Choi et al., 2007), most reports maintain that CHIP activity is, by and large, neuroprotective. It is, therefore, important to elucidate the mechanisms by which this ligase, as well as other the components of the system function, and how they may be harnessed for use in disease therapeutics.

While overexpressing CHIP is one potential therapeutic route, a better understanding of its regulation could lead to the discovery of potential drug targets for upregulating protein quality control activity in other ways. Previous work on various RING family ubiquitin ligases has shown that they can be regulated via many routes, including phosphorylation, protein-protein interactions, and additions of small ubiquitin-like molecules such as Nedd8 and SUMO-1 (Joazeiro & Weissman, 2000). Such modifications can either inhibit or enhance ligase activity, and in some cases might alter localization as well (Joazeiro & Weissman, 2000). The RING finger E3 Mdm2 not only ubiquitinates substrates, but also mediates the attachment of ubiquitin to itself (a process hereon referred to as autoubiquitination), thereby targeting itself for proteasomal degradation (Fang et al., 2000). Not all E3 autoubiquitination serves to target the ligase for degradation, however. Autoubiquitination was observed with the heterodimeric RING

finger ligase BRCA1-BARD1 (often implicated in breast and ovarian cancer), whereby ubiquitin chains were formed on BRCA1-BARD1 complex (Chen et al., 2002). These chains contain non-K48 linkages, and do not serve to target the complex for degradation (Chen et al., 2002). Instead, the ubiquitin chains on the ligase might serve as a signaling platform involved in DNA repair, as that is a significant function of BRCA1 (Chen et al., 2002).

As mentioned above, the pairing of particular E2 enzymes with E3 ligases is a major determinant of the consequences of the ubiquitination. Therefore, we begin by describing CHIP's functional association with a group of ubiquitin conjugating enzymes (E2 enzymes) with which it is predicted to interact, based on the fact that these enzymes contain a critical serine-proline-alanine (SPA) motif, described by Xu et al. (2008). We have assessed and compared their ability to work together with CHIP and ubiquitinate a model substrate. Additionally, we have examined the ability of multiple E2s to work together in conjunction with CHIP. Prior studies with various E3s have suggested that E2 enzymes from two different classes collaborate: the first attaches a single ubiquitin, while the other elongates the chain (Rodrigo-Brenni, 2007; Christensen et al., 2007; Windheim et al., 2008). This has been observed both in the case of autoubiquitination of the ligase (Christensen et al., 2007; Windheim et al., 2008), as well as with ubiquitination of a model substrate (Rodrigo-Brenni et al., 2007). Certain E2 enzymes are unable to ubiquitinate substrates, and in absence of an acceptor ubiquitin, they merely form unanchored ubiquitin chains (Christensen et al., 2007; Windheim et al., 2008). The studies agree that after one E2 attaches an "acceptor" ubiquitin, the second E2 – often the type that forms free chains on its own – catalyzes the formation of ubiquitin chains with a specific linkage type (either K48 or K63 linked), a specificity that is dependent upon the E2 (Rodrigo-Brenni, 2007; Christensen et al., 2007; Windheim et al., 2008). Although Windheim et al (2008) performed

their study using CHIP as the ligase in question, they examined only its autoubiquitination.
Nonetheless, the idea of sequential E2 action in the ubiquitination of a misfolded substrate has
been proposed by Xu et al. (2008). In light of the prior findings, we have examined the ability of
pairs of E2 enzymes to act with CHIP to ubiquitinate a misfolded model substrate and found that
such sequential action does indeed appear to take place.

Furthermore, unpublished data from our laboratory has shown that one particular E2
autoubiquitinates CHIP with particularly great efficiency. As previously discussed,
autoubiquitination of E3 ligases has been observed in a few cases, either targeting them for
degradation or serving as a putative signaling platform (Fang et al., 2000; Chen et al., 2002). In
hopes of elucidating the significance of CHIP autoubiquitination, we have worked on various
binding assays with known interactors of CHIP. One such experiment involves the differential
binding of proteins with ubiquitin interacting motifs (UIMs) to ubiquitinated versus unmodified
CHIP. We show that the proteasome subunit S5a, which contains two UIMs, preferentially binds
to ubiquitinated CHIP. The function of this is still unclear, but might perhaps play a role in
shuttling to the proteasome (Wang et al., 2005). Also among the UIM-containing proteins
recruited to autoubiquitinated CHIP is the de-ubiquitinating enzyme ataxin-3. When it contains
an expanded polyglutamine stretch over the threshold of about 50 glutamine residues, it is the
pathogenic species in SCA3 (Warrick et al., 2005). However, even in its wild-type form, ataxin-
3 is suspected to play a significant role in the ubiquitin-proteasome system. In its amino-
terminus, ataxin-3 contains a catalytic domain (termed the Josephin domain), and its carboxy-
terminus contains three UIMs (Winborn et al., 2008). Prior work has shown that ataxin-3
preferentially binds ubiquitin chains of four or more units – the ideal chain length for
proteasomal targeting – through its UIMs, and its catalytic domain acts as a ubiquitin protease to

remove ubiquitin from polyubiquitin chains (Burnett et al., 2003). Furthermore, ataxin-3 has been shown to preferentially cleave K63-linked chains, thereby providing a mechanism to edit chains (Winborn et al., 2008). Alternatively, chain editing might occur via simply positioning ubiquitin molecules in a particular conformation to promote one linkage type over others. Such chain editing activity might assist in formation of ubiquitin chains that can target proteins for degradation by the proteasome. Without this editing, CHIP paired with the E2 enzyme UbcH5c (Ube2d3) has been shown to make branched mixed linkage polyubiquitin chains that cannot be degraded by the proteasome (Kim et al., 2007).

In the results that follow, we show that the ubiquitin conjugating enzyme Ube2w efficiently ubiquitinates CHIP, which in turn recruits UIM-containing proteins such as ataxin-3. Ataxin-3, in turn, trims chains and facilitates proteasomal degradation of misfolded substrate. Finally, we examine various UIM mutant forms of ataxin-3 and their performance in CHIP-mediated ubiquitination assays, and show that the first two UIMs are critical for its chain-trimming ability.

Materials and Methods

Protein purification

Proteins were purified from *E. coli* BL21 cells via affinity purification using glutathione beads for GST fusion proteins (ataxin-3 and its mutants, CHIP, Ube2z) or nickel beads for 6X histidine tagged proteins (all other E2 enzymes). GST fusion proteins were cleaved from their GST tag with PreScission Protease (GE Life Sciences). GST fusion proteins were resuspended and stored in phosphate buffered saline, while His-tagged proteins were resuspended and stored in a Tris-based buffer (50 mM) with NaCl (0.1 M) and imidazole (20 mM). Proteins were subjected SDS-PAGE and stained with Coomassie blue in order to determine protein concentration.

Ubiquitination Assays

In vitro ubiquitination studies were carried out in a 50 mM Tris HCl buffer (pH 7.5), with 50 mM KCL and 0.2 mM DTT. Unless otherwise stated, the reaction mixture included 1 μM each of E2 and E3 (CHIP), 50 nM E1, 20 μM ubiquitin, 2.5 mM ATP and 2.5 mM $MgCl_2$ in a total reaction volume of 10 μl. If the model substrate was the chaperone Hsp90, 1 μM was used. If recombinant luciferase (rLuc) served as the model substrate, such as in the E2:E3 pairing assays, 0.14 μg rLuc was mixed with 1 μM of Hsp90 and buffer, and incubated at 42° C for 7 minutes in order to denature the luciferase and was subsequently included in the reaction mixture. In assays where ataxin-3 (either wild type or mutant form) was used, it was present at a 1 μM concentration. When proteasomal degradation was assessed, purified 26S proteasome (Boston Biochem) was included at a 10 nM concentration. Reactions were allowed to run at 37° C for various timepoints (as stated in each figure), stopped with Laemmli buffer (0.24 M Tris pH 6.8,

0.08% SDS, 40% glycerol, 0.02% bromophenol blue, 10% ß-mercaptoethanol, water) and boiled for 4 minutes at 100° C.

Immunoblotting

Reaction mixtures were run on SDS-PAGE gels (10%, 15%, or 17.5%) and transferred to Polyvinylidene fluoride (PVDF) membranes. Western blots were performed with the following antibodies in 5% milk in TBS-t: goat anti-luciferase (concentration 1:1000), rabbit anti-CHIP (1:1000), mouse anti-Hsp90 (1:1000), mouse anti-ataxin-3 (MJD or 1H9; 1:25000 and 1:1000, respectively), mouse anti-ubiquitin (1:5000), mouse anti-UbcH5 (1:1000), and rabbit anti-His (1:500). The appropriate secondary antibodies (goat anti-mouse, goat anti-rabbit, or donkey anti-goat) conjugated to horseradish peroxidase were used at a concentration of 1:12500. Blots were developed using Western Lightning chemiluminescence reagent (Perkin Elmer).

Binding Assay

A ubiquitination reaction was carried as described above for 90 minutes using Ube2w as the E2, though without the presence of either Hsp90 nor rLuc, and using CHIP with a glutathione S-transferase (GST) affinity tag. A control reaction was performed lacking ubiquitin. The mixture was then added to 50 μl of glutathione beads and allowed to bind overnight at 4° C. Unbound proteins were then washed away with NETN buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP40) with 0.5 mM DTT. 1 μM of ataxin-3 or S5A was added along with NETN buffer (to total 20 μl) to a 20 μl volume of either empty beads, beads with CHIP, or beads with ubiquitinated CHIP. The proteins were allowed to bind for 1 hour at 4° C, after which any excess was washed with NETN + DTT. The bound proteins were eluted with 20 μl of 1x Laemmli and boiled for 4 minutes. They were then subjected to 10% SDS-PAGE followed by Western blot, as described above.

Results

CHIP acts with a variety of ubiquitin conjugating enzymes

CHIP was sequentially matched with members a cohort of E2 enzymes and the pairs were tested for their ability to ubiquitinate Hsp90 or heat-denatured luciferase (rLuc) as well as CHIP itself (Figure 1).

All four E2 enzymes in the Ube2d (UbcH5) family robustly polyubiquitinate both rLuc and Hsp90, as evidenced by the presence of high molecular weight species that accumulate throughout the course of the reaction. The enzymes in this group are all roughly equal in their ability to ubiquitinate substrate (the differences in darkness observed in the blots are largely attributable to exposure differences). Polyubiquitination of rLuc was also observed with Ube2e1 (UbcH6), though only at later time points (15-90 minutes) and to a lesser extent than with UbcH5 family E2s. Meanwhile, Ube2w rapidly and efficiently monoubiquitinated rLuc, and had no significant activity with Hsp90 (the slight monoubiquitination of Hsp90 is extremely limited and only observed at very late time points). Monoubiquitination of rLuc and Hsp90 is seen with Ube2e1 (UbcH6) at earlier time-points, and this enzyme appears to be more adept at monoubiquitination than polyubiquitination. In the meantime, Ube2z (Use1) does not seem to cooperate with CHIP to ubiquitinate rLuc. It is important to note, however, that this protein could only be purified with an attached glutathione S-transferase (GST) tag, and repeated attempts to purify the cleaved protein were unsuccessful. Thus, the lack of activity may be attributed to a steric hindrance caused by the bulky epitope tag. Finally, the heterodimer Ubc13/Mms2 has previously been shown to form free polyubiquitin chains with CHIP (Christensen et al., 2007).   Our results show no mono or di-ubiquitination of rLuc or Hsp90. The rLuc blot shows some high molecular weight species trapped in the stacking gel, which are

consistent with polyubiquitin chains made by Ubc13 and afterwards attached to rLuc. Hsp90 is

not ubiquitinated by Ubc13.

Autoubiquitination of CHIP was also noted with each E2. The four enzymes in the

Ube2d family, as well as Ube2e1, all showed autoubiquitination of CHIP to some extent, though

there was still a large amount of unmodified CHIP present. None of these effects were as

dramatic as that observed with Ube2w, however, which functioned to monoubiquitinate CHIP

swiftly and completely. By five minutes, the vast majority of CHIP is ubiquitinated, and

unmodified CHIP disappears entirely by 30 minutes. Presence or absence of rLuc has no effect

on this ability (data not shown).

Ube2w cooperates with other ubiquitin conjugating enzymes

When tested for ubiquitination of recombinant luciferase, Ube2w alone is observed to

monoubiquitinate the substrate to a significant extent, while UbcH5c is able to polyubiquitinate

it. When the two E2 enzymes are combined an enhancement of ubiquitination activity is

observed, with more extensive polyubiquitination of rLuc, particularly at the 10 minute time-

point (Figure 2A). A shorter exposure of the blot emphasizes that extensive monoubiquitination

of rLuc is seen only when Ube2w is present in the reaction. In a separate experiment with only

Hsp90 present as a model substrate, monoubiquitination is not observed with Ube2w, suggesting

that Hsp90 is not an ideal substrate for this E2 (Figure 2B). When both of Ube2w and UbcH5c

are present together, Hsp90 is polyubiquitinated. However, UbcH5c is able to polyubiquitinate it

without any additional E2 enzymes, and no enhancement of this activity is observed when

Ube2w and UbcH5c are combined. In fact, there actually appears to be a smaller amount of high

molecular weight species (polyubiquitinated Hsp90) when the E2 enzymes are combined, presumably because they compete for binding to CHIP.

In accordance with the previous findings, when Ube2w is combined with Ubc13, a new activity emerges that is not observed with either E2 on its own: together, the enzymes are able to form ubiquitin chains on rLuc (Figure 3). As mentioned above, Ube2w only attaches a single ubiquitin rLuc. Meanwhile, Christensen et al. (2007) have previously shown that Ubc13 on its own forms unanchored K63-linked polyubiquitin chains, and as such, we did not observe rLuc ubiquitination using Ubc13 alone. With the inclusion of both Ube2w and Ubc13, however, rLuc was polyubiquitinated. Together, the two enzymes also polyubiquitinate CHIP – this, too, is not seen with either enzyme acting alone.

Ube2w robustly monoubiquitinates CHIP

In a standard ubiquitination reaction, Ube2w is found to quickly and efficiently monoubiquitinate CHIP (Figure 4A). Meanwhile, UbcH5c is found to very weakly monoubiquitinate CHIP, an activity observed only at later time points, which contrasts sharply with the rapid activity of Ube2w.

Ube2w was also tested with CHIP K22R, a mutant form of the ligase that contains a lysine to arginine point mutation in the lysine where it is normally ubiquitinated. Additionally, previous work in our laboratory has shown that this mutant form is unable to support Hsp90 ubiquitination (with UbcH5c as the E2) under our experimental conditions. We found, however, that Ube2w can still monoubiquitinate CHIP K22R, suggesting that there is an alternative lysine at which CHIP can be ubiquitinated (Figure 4B). Based on the amount substrate disappearance, the mutant form is not ubiquitinated as completely as wild type CHIP. In attempts to find out if

pre-ubiquitinating CHIP K22R might restore its ubiquitin ligase activity (when paired with

UbcH5c), ubiquitination assays were carried out in which CHIP K22R was first incubated with

Ube2w and the other necessary components of the ubiquitination assays to facilitiate its

monoubiquitination, and was followed an hour later by UbcH5c and Hsp90. Our results showed,

however, that despite being pre-ubiquitinated, CHIP K22R was still unable to ubiquitinate

Hsp90, unlike its wild-type counterpart (Figure 4C). This suggests that lysine 22 has particular

importance and must be intact in order to mediate substrate ubiquitination, and the alternate

lysine is not sufficient.

Furthermore, in attempt to better characterize Ube2w, recent experiments in our

laboratory have suggested that Ube2w may have more than one active site (Scaglione et al.,

unpublished data). In an attempt to verify this possibility, Ube2w and Ube2w C91A (a form of

the E2 carrying a point mutation in its putative active site cysteine) were each incubated with the

necessary components of a ubiquitination reaction, as described in Methods. The mutant Ube2w

was found to be inactive in ubiquitinating rLuc and CHIP, thus suggesting that the cysteine at

residue 91 is necessary for Ube2w's activity as a ubiquitin conjugating enzyme (Figure 4D).

Autoubiquitinated CHIP recruits UIM-containing proteins

To elucidate the function of CHIP autoubiquitination, we tested the ability of monoubiquitinated

CHIP to recruit UIM-containing proteins. CHIP fused to glutathione S-transferase (GST) was

pre-ubiquitinated with Ube2w, and bound to glutathione beads. Immunoblotting confirms that

GST-CHIP was indeed monoubiquitinated by Ube2w (Figure 5), though not as robustly as

untagged CHIP in previous experiments, possibly because of a steric hindrance due to the bulky

GST tag. As described in the experimental methods, empty beads, unubiquitinated GST-CHIP,

or pre-ubiquitinated CHIP was incubated with the proteasomal subunit S5a or the deubiquitinating enzyme ataxin-3. S5A was found to bind successfully to monoubiquitinated CHIP, and comparison with the input control revealed that approximately 10% of the S5A that was initially added to the binding reaction was recovered (Figure 5). Ataxin-3 yielded similar results, with more ataxin-3 bound to monoubiquitinated than to unmodified CHIP.

Ataxin-3 plays a role in the trimming of polyubiquitin chains, facilitating degradation

As our results show that UIM-containing proteins such as ataxin-3 are recruited to monoubiquitinated CHIP, there was considerable interest in determining their role in CHIP-mediated ubiquitination reactions in order to better understand their function as a part of the UPS. Work by others in our laboratory has shown that ataxin-3 works to cleave long polyubiquitin chains on substrate proteins (Scaglione et al., in preparation). An immunopurification experiment against rLuc that had been ubiquitinated in the presence or absence of ataxin-3 is shown in Figure 6A. First, blotting for ubiquitin confirms that the higher molecular weight species formed in the absence of ataxin-3 are indeed highly polyubiquitinated forms of rLuc, as opposed to merely aggregated protein. Furthermore, reactions without ataxin-3 resulted in longer ubiquitin chains on the substrate protein (as shown by the higher molecular weight species) than reactions with ataxin-3. This result is corroborated by a reaction in which Hsp90 is used as the model substrate (Figure 6B). Without ataxin-3, a tight band of highly ubiquitinated Hsp90 is seen at the top of the gel. In the presence of ataxin-3, however, the molecular weight of this band is noticeably decreased.

Next, data gathered by Matthew Scaglione in our laboratory demonstrates that when a 26S proteasome is included in the reaction mixture, the presence of ataxin-3 facilitates the

degradation of ubiquitinated substrate, as indicated by the gradual disappearance of rLuc in later time-points (Figure 6C). Furthermore, it is worth noting that CHIP is gradually deubiquitinated in the presence of ataxin-3 as the reaction progresses and substrate is depleted. Such deubiquitination is not observed in the absence of ataxin-3.

UIMs 1 and 2 of ataxin-3 are critical for chain-trimming

In order to hone in on the domains of ataxin-3 that are critical for its chain-editing functions, assays were carried out with various mutant forms of the protein. Mutants carrying a C14A mutation in the active site of the catalytic domain did not exhibit chain-trimming activity, as expected (data not shown). Other mutants carried two point mutations (alanine to serine and glycine to aspartic acid) in one or more of their UIMs, which in turn greatly diminishes their ability to bind ubiquitin. In the ubiquitination assays, Hsp90 served as the model substrate, and UbcH5c as the E2. Without ataxin-3 in the reaction, Hsp90 was ubiquitinated to a great extent, forming a tight band at the top of the gel that is likely to correspond to Hsp90 with long ubiquitin chains attached, in accordance with data cited above. In the presence of wild-type ataxin-3, chain-trimming is once again observed. On the other hand, the reduction in chain length is not observed with the UIM 1 mutant, UIM 2 mutant, UIM 1 & 2 mutant, or UIM 1, 2 & 3 mutant forms of ataxin-3 (Figure 7). Mutating UIM 3 alone, however, still allows for shorter ("trimmed") chains.

Discussion

Testing the activity of CHIP with various ubiquitin conjugating enzymes has shown that they vary in their effectiveness in ubiquitination of a misfolded protein substrate. While some, such as the members of the Ube2d (UbcH5) family, are able to polyubiquitinate the substrate, others, particularly Ube2w, merely monoubiquitinate it. Work by prior groups has suggested that some E2s may work in concert, the first attaching a single ubiquitin and the other extending the chain (Rodrigo-Brenni, 2007; Christensen et al., 2007; Windheim et al., 2008). Using Ube2w paired with either UbcH5c or Ubc13/Mms2, we showed that this phenomenon occurs in CHIP-mediated ubiquitination reactions as well. The effect is most dramatic with Ubc13, as this E2 does not ubiquitinate substrates on its own (Christensen et al., 2007), but is able to extend chains on a substrate protein that has been pre-ubiquitinated with Ube2w. Although UbcH5c is able to polyubiquitinate substrate on its own, our results indicate that its polyubiquitinating activity is enhanced when the substrate is first monoubiquitinated.

Prior work from groups working with ubiquitin ligases other than CHIP reveals an intriguing correlation with relevance to E2 cooperation: E2 enzymes that are able to noncovalently bind ubiquitin at a site distal from the active site are the same E2s that are able to form polyubiquitin chains (Christensen et al., 2007; Rodrigo-Brenni, 2007). Among this group are Ubc13 and UbcH5c (Christensen et al., 2007), which agrees with our above findings. It would be of great interest for us to verify this correlation by testing a panel of all known E2 enzymes for ubiquitin-binding ability via surface plasmon resonance, and examine their performance in *in vitro* ubiquitination assays. Thus, we could theoretically split E2 enzymes into two categories: those that monoubiquitinate substrates, and others that extend chains on the

acceptor ubiquitin. This, in turn, might lead us closer to identifying targets for upregulation to help cells better cope with proteotoxic stress.

Upon examining the ubiquitination of reaction components other than the misfolded substrate, we discovered the function of Ube2w in monoubiquitinating CHIP. This is an exciting and novel result, as no E2 studied previously has ubiquitinated CHIP to such a great extent and done so as quickly. Naturally, this leads to questions about the role of CHIP autoubiquitination. Other researchers in the laboratory have shown that Ube2w is upregulated during times of cellular stress, suggesting that its unique activity (of ubiquitinating CHIP) plays an important role in the cellular response to stress (Scaglione et al., in preparation).

The binding assays described above help uncover the purpose of CHIP autoubiquitination. The differential binding of S5a and ataxin-3 to monoubiquitinated CHIP (as opposed to unmodified CHIP) suggests that the role of the single ubiquitin on CHIP is to recruit UIM-containing proteins to the ubiquitination reaction. Additional work can be done to confirm this result with additional UIM-containing proteins.

One of the UIM-containing proteins recruited by monoubiquitinated CHIP, ataxin-3, has shown chain-trimming activity in a ubiquitination reaction. This function, however, depends on it having an intact active site and intact UIM 1 & 2. It appears that UIM 3 is less important for this purpose. The results are consistent with the finding that ataxin-3 preferentially cleaves longer chains (Burnett et al., 2003; Winborn et al., 2008). This chain-trimming facilitates targeting of misfolded substrates to the proteasome, as the ideal chain length for proteasomal targeting is approximately four ubiquitins (Burnett et al., 2003). Results from our laboratory confirm that the presence of ataxin-3 enhances the proteasomal degradation of a heat-denatured protein substrate. Furthermore, as previously mentioned, ataxin-3 has been shown to edit

ubiquitin chains, preferentially cleaving K63 linkages, thus specifying the type of linkages that are present in ubiquitin chains on the substrates in question (Winborn et al., 2008). This, too, might be a potential mechanism by which recruitment of ataxin-3 assists in the formation of chains of the proper type (4-5 ubiquitins in length, typically linked via lysine 48) to target substrates for proteasomal degradation. Consequently, pieces of a model begin to fall into place: Ube2w monoubiquitinates CHIP, which recruits UIM-containing proteins such as ataxin-3 that modify the kinds and lengths of ubiquitin chains present on the misfolded protein substrates, thus assisting in their targeting to the proteasome for degradation (Figure 8).

In light of our proposed model, it comes as no surprise that wild-type unexpanded ataxin-3 has been shown to have a neuroprotective function and suppress polyglutamine neurodegeneration *in vivo* (Warrick et al., 2005). Drosophila expressing wild-type ataxin-3 along with the expanded form exhibited less disease protein accumulation as well as reduced retinal neurodegeneration (Warrick et al., 2005). Such neuroprotective effects are dependent on a functional proteasome and intact protease domain of ataxin-3, while mutating its UIMs also compromises its protective function (Warrick et al., 2005). These findings lend support to our model, which suggests that ataxin-3 is recruited to reactions via its UIMs, and its activity contributes to proteasomal targeting of substrates. Hence, abolishing either proteasomal function or ataxin-3's UIMs would eliminate any beneficial effects that it might have in assisting the elimination of misfolded protein species.

The literature reveals another case that might bear considerable resemblance to CHIP autoubiquitination. Mutations in the parkin protein have been implicated in certain familial forms of Parkinson's disease, particularly the early-onset variety (Sriram et al., 2005). Like CHIP, parkin is a cytosolic RING-type E3 ubiquitin ligase, and has been found to function in

ERAD (ER-associated degradation) and in cytosolic quality control (Cyr et al., 2002). It differs from CHIP, however, in that it contains a ubiquitin-like domain (UBL). We suspect that this UBL might be analogous to the single ubiquitin attached to CHIP by Ube2w. A particular mutation (R42P) in the UBL of parkin was found to alter parkin's ubiquitin properties: specifically, the chains it forms on the substrate synphilin-1 are longer than those formed with wild type parkin (Sriram et al., 2005). Subsequently, these substrates cannot be successfully degraded by the proteasome. The authors suggest that this might be due to impaired binding of mutant parkin to the proteasome (Sriram et al., 2005). We, however, propose that a mutation in the ubiquitin-like domain may impair the recruitment and binding of UIM-containing proteins to parkin, thus not providing the crucial chain-editing activity that is necessary for the formation of proper chain lengths and linkage types and consequent targeting to the proteasome. Further work is necessary to test this hypothesis: a binding assay similar to the one performed with monoubiquitinated CHIP would likely be a useful initial experiment. Nonetheless, the possibility exists that our model might extend to other E3 ligases, including ones implicated in neurodegeneration.

Overall, the work presented here reveals a model to describe the possible mechanism of CHIP-mediated ubiquitination reactions. A better understanding of this mechanism has significant implications when viewed from a therapeutic standpoint: for example, instead of focusing exclusively on upregulation of CHIP itself, we might broaden our scope to include other players in the UPS, such as Ube2w. Before that point, however, the model proposed above must be verified *in vivo*. Although *in vitro* ubiquitination reactions allow for well-controlled experiments that examine the "bare bones" mechanism, the physiological relevance of these findings must be assessed. This could be done first through cell culture, followed by mouse

models. For example, mice with Ube2w either knocked out or overexpressed might be expected to show decreased and increased abilities to cope with proteotoxic stress (respectively). Thus, the results presented above help lay the groundwork for the identification of potential targets of neurodegenerative disease therapeutics.
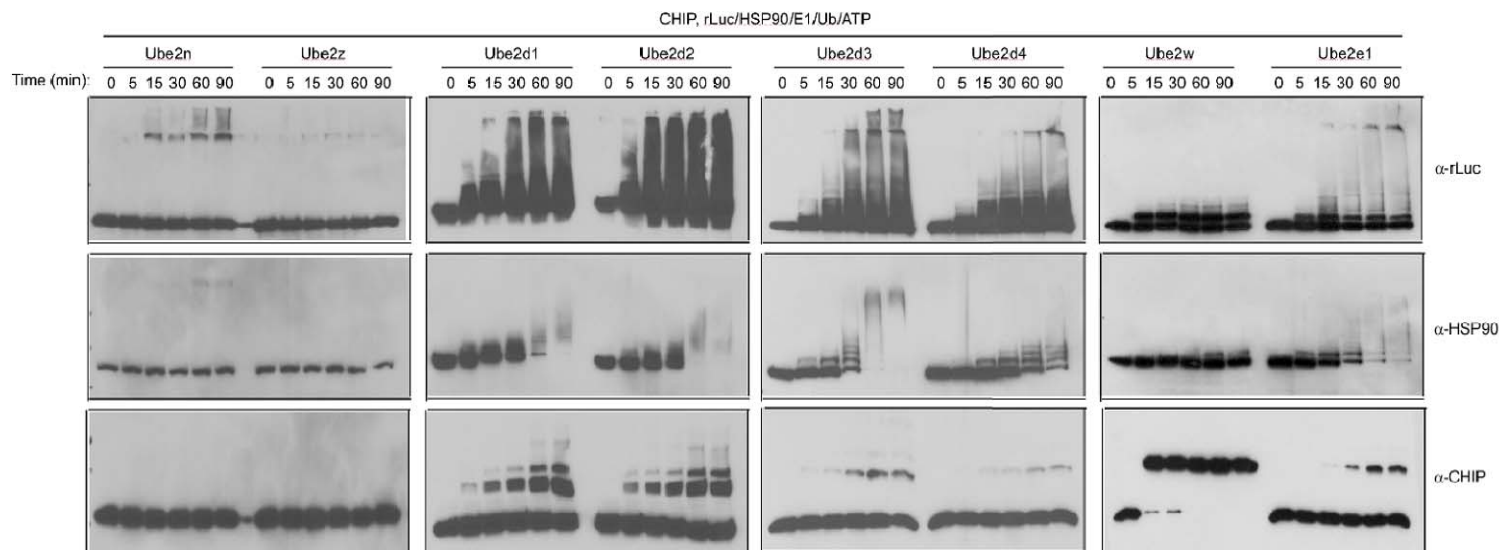
Figure 1. CHIP acts with a variety of ubiquitin conjugating enzymes
1 µM CHIP was incubated with 1 µM of E2 as indicated, 50 nM E1, 20 µM ubiquitin, 2.5 mM ATP and 2.5 mM MgCl$_2$, as well as 0.14 µg heat-denatured luciferace and 1 µM of Hsp90. E2 enzymes of the Ube2d (UbcH5) family were observed to polyubiquitinate rLuc, while Ube2w attaches a single ubiquitin. Ube2e1 (UbcH6) is effective at rLuc monoubiquitination, but shows limited polyubiquitination activity as well. Ube2n and Ube2z do not appear to attach ubiquitin directly to substrate. Enzymes of the Ube2d family also polyubiquitinate Hsp90, Ube2e1 monoubiquitinates the chaperone, and Ube2w does not significantly modify it. CHIP ubiquitination is also observed to varying degrees, ranging from robust monoubiquitination by Ube2w to no modification by Ube2n (Ubc13).
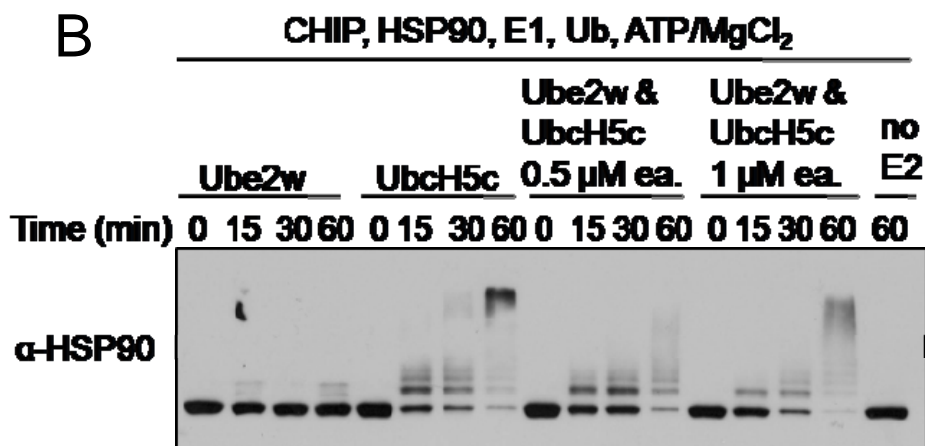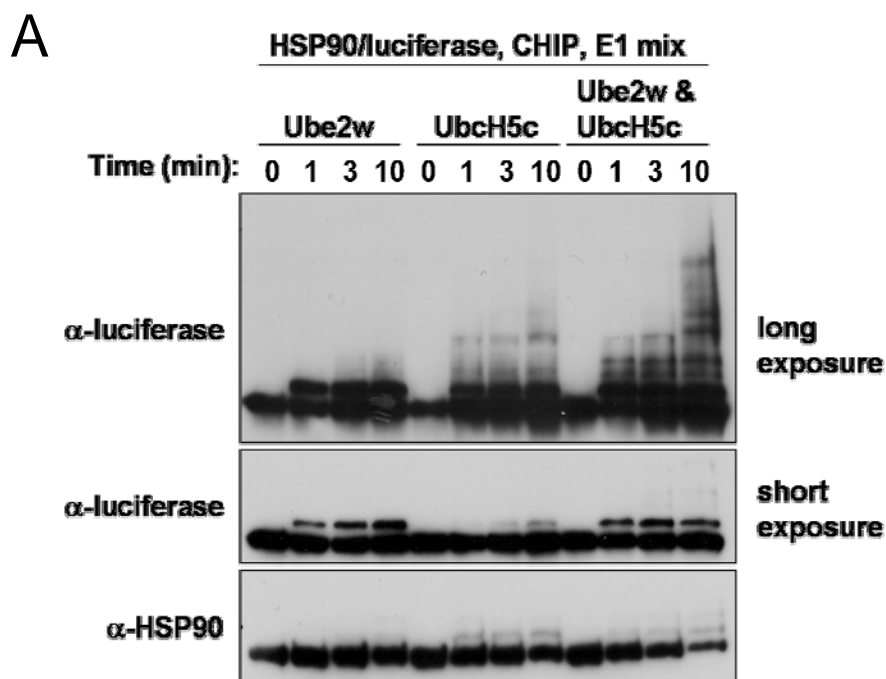
A

HSP90/luciferase, CHIP, E1 mix

	Ube2w	UbcH5c	Ube2w & UbcH5c
Time (min):	0 1 3 10	0 1 3 10	0 1 3 10

α-luciferase — long exposure

α-luciferase — short exposure

α-HSP90

B

CHIP, HSP90, E1, Ub, ATP/MgCl$_2$

	Ube2w	UbcH5c	Ube2w & UbcH5c 0.5 µM ea.	Ube2w & UbcH5c 1 µM ea.	no E2
Time (min)	0 15 30 60	0 15 30 60	0 15 30 60	0 15 30 60	60

α-HSP90

Figure 2. Ube2w cooperates with UbcH5c
Ubiquitination reactions were carried out as previously described, though with the inclusion of two E2 enzymes, Ube2w and UbcH5c (Ube2d3). **(A)** Ubiquitination of rLuc is enhanced with the presence of both E2s as compared to either E2 on its own. **(B)** A similar reaction was carried out without rLuc, thus Hsp90 served as a model substrate. No enhancement of Hsp90 ubiquitination was observed with both E2s present.
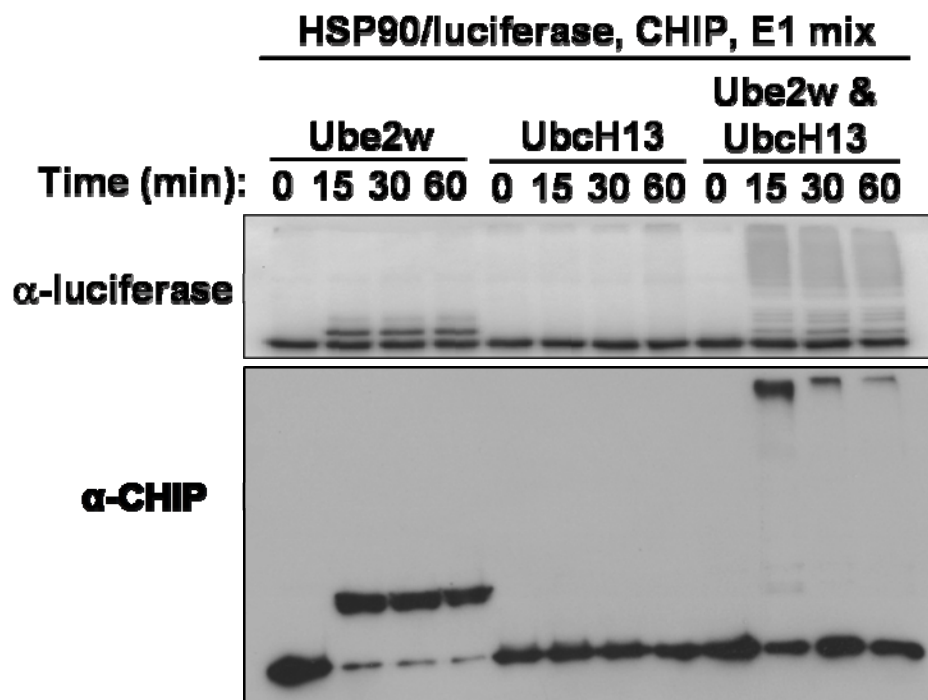
Figure 3. Ube2w cooperates with Ubc13 to form anchored polyubiquitin chains on substrate
Representative blots from ubiquitination assays using heat-denatured luciferase as the model substrate. While Ube2w monoubiquitinates rLuc, Ubc13 (or its human homologue, UbcH13) does not ubiquitinate this substrate. Combining the two E2 enzymes in equimolar amounts resulted in extensive ubiquitination of rLuc. CHIP monoubiquitination is observed with Ube2w, but not Ubc13. Polyubiquitinated CHIP emerges when both E2s are used.
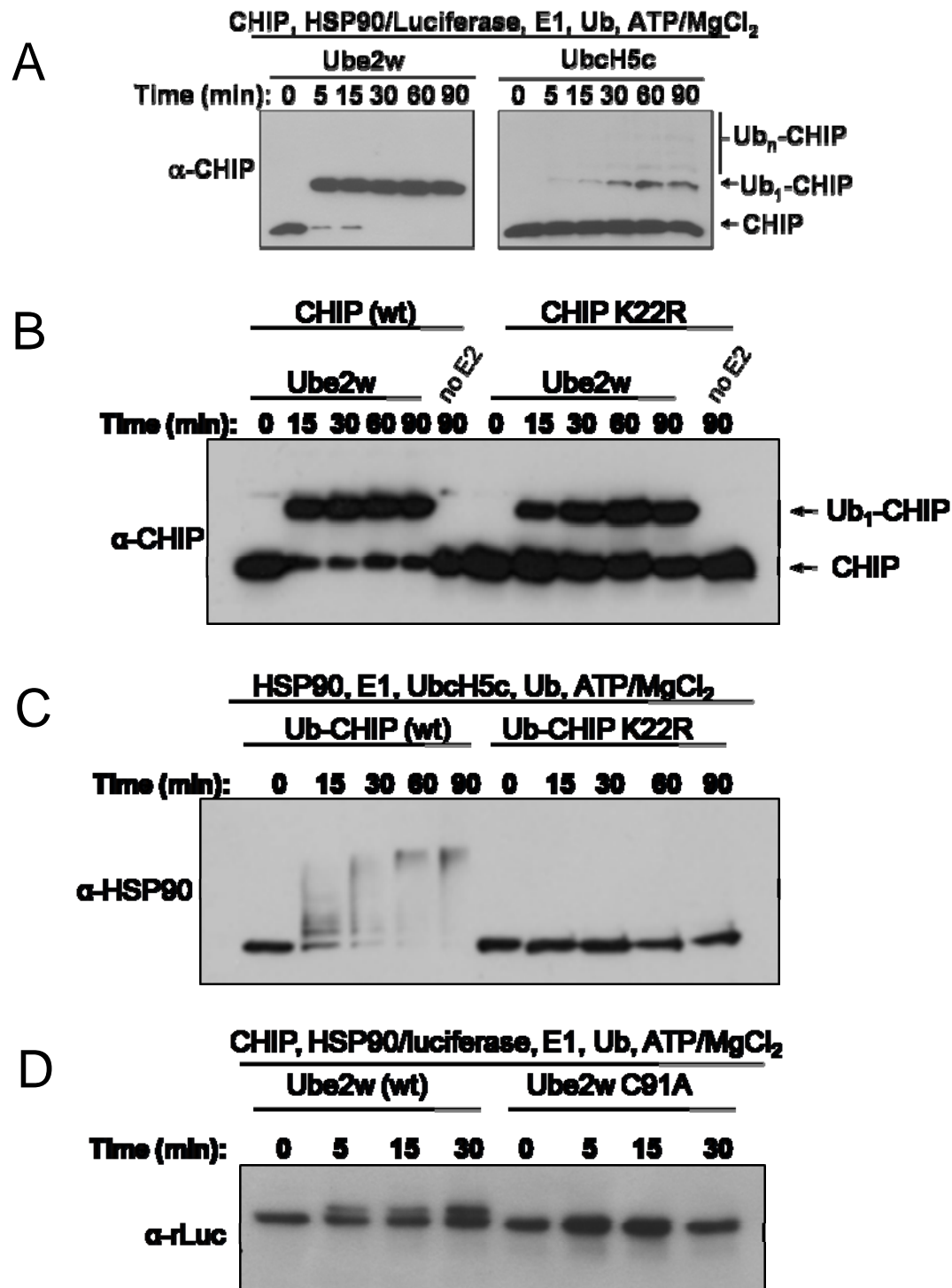
Figure 4. Ube2w robustly ubiquitinates CHIP as well as a CHIP mutant
(A) Comparison of CHIP autoubiquitination in the presence of two different E2 enzymes. Ube2w rapidly and efficiently monoubiquitinates CHIP, in contrast with UbcH5c (Ube2d3).
(B) In addition to monoubiquitinating wild-type CHIP, Ube2w is also able to monoubiquitinate a CHIP mutant lacking the lysine thought to be the site of monoubiquitination.
(C) CHIP was incubated with Ube2w, E1, ubiquitin, and ATP/MgCl$_2$ for one hour to obtain ubiquitinated CHIP, as in (B). Subsequently, UbcH5c and Hsp90 (as the model substrate) were added, and the reaction was allowed to run for the times indicated. Hsp90 ubiquitination was observed with wild-type CHIP, but not the CHIP K22R mutant.
(D) Ube2w carrying a mutation in its putative active site cysteine is inactive in ubiquitinating rLuc.
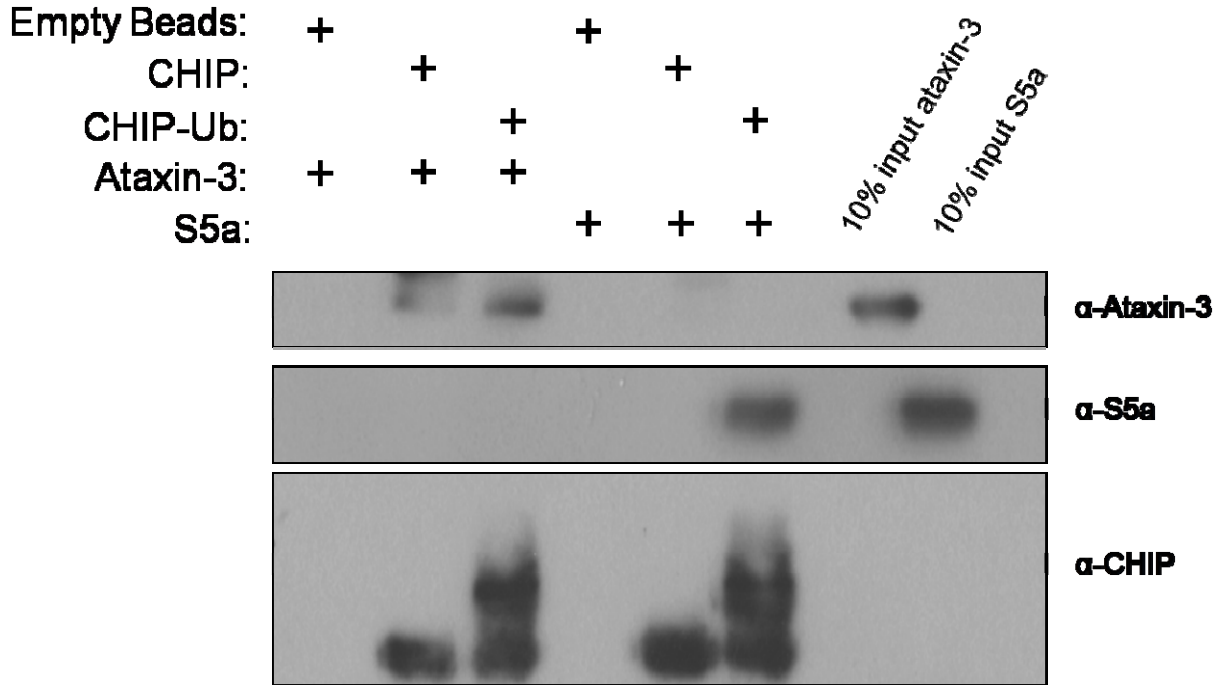
Figure 5. CHIP autoubiquitination recruits proteins containing ubiquitin interacting motifs (UIMs)

GST-CHIP was incubated with Ube2w, E1, Ub, and ATP/MgCl$_2$, then bound to glutathione beads, and subsequently incubated with ataxin-3 or S5a. The beads were then washed and bound proteins eluted. S5a binds to ubiquitinated, but not unmodified CHIP. Ataxin-3 also binds to a greater extent to ubiquitinated CHIP. The final two lanes contain 10% of the original input, shown for the purpose of comparison.
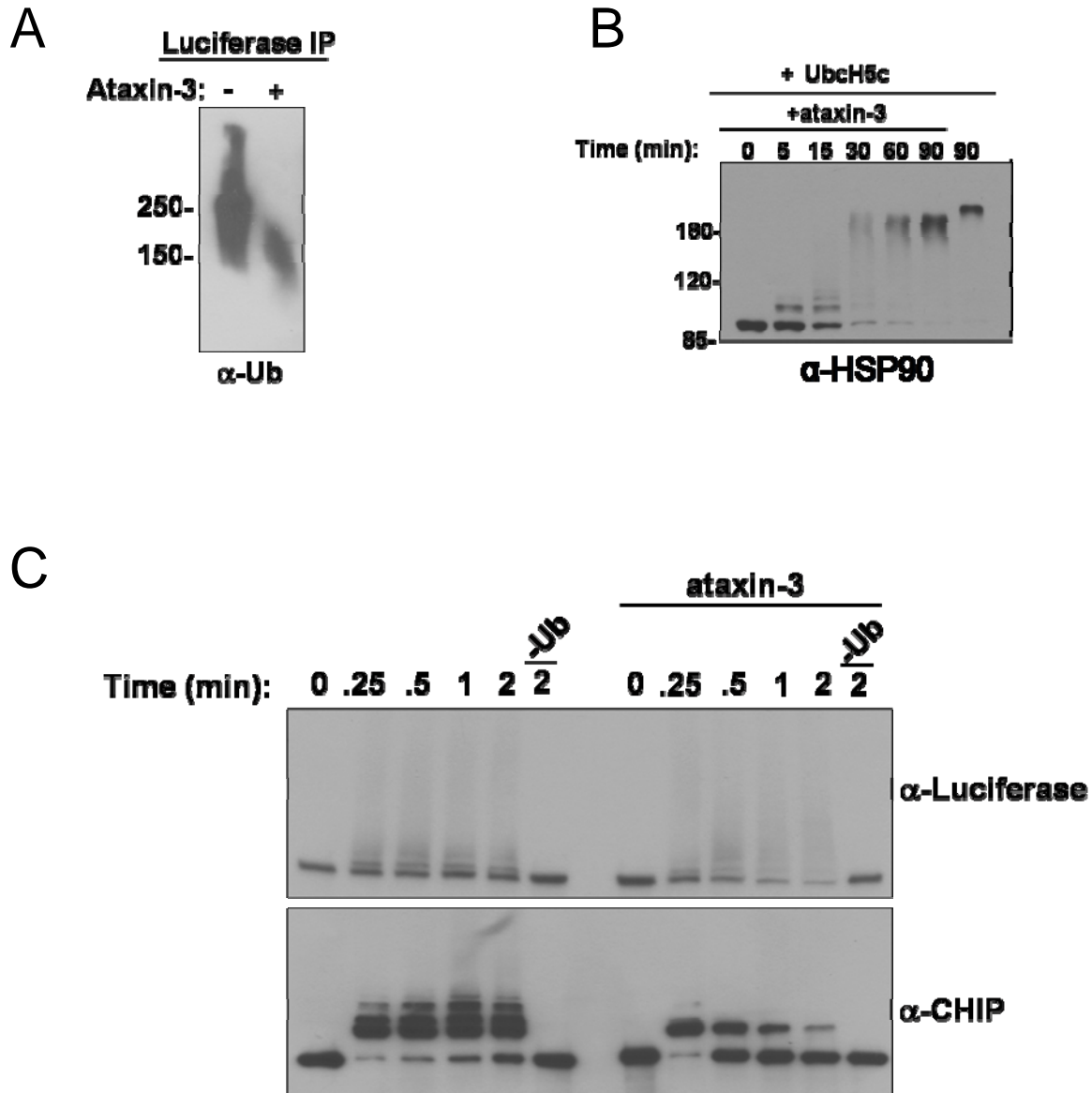
Figure 6. Ataxin-3 trims polyubiquitin chains and facilitates proteasomal degradation of ubiquitinated substrate

(A) Heat-denatured luciferase was incubated with Hsp90, CHIP, E1, Ube2w, UbcH5c, ATP/MgCl2, in the presence or absence of ataxin-3, and subsequently immunoprecipitated with antibodies against luciferase bound to IgG beads. More extensive polyubiquitination of rLuc is observed in the absence of ataxin-3.

(B) A ubiquitination reaction was carried out as previously described with only UbcH5c as the E2, and Hsp90 as the model substrate. When ataxin-3 is present in the reaction, the polyubiquitinated rLuc species have a lower molecular weight then they do when ataxin-3 is absent.

(C) rLuc, Hsp90, CHIP, E1, Ube2w, UbcH5c, and ATP/MgCl2 were incubated with 26S proteasome in the presence or absence of ataxin-3. Degradation of rLuc is enhanced in the presence of ataxin-3. CHIP is also deubiquitinated as the reaction progresses when ataxin-3 is included.
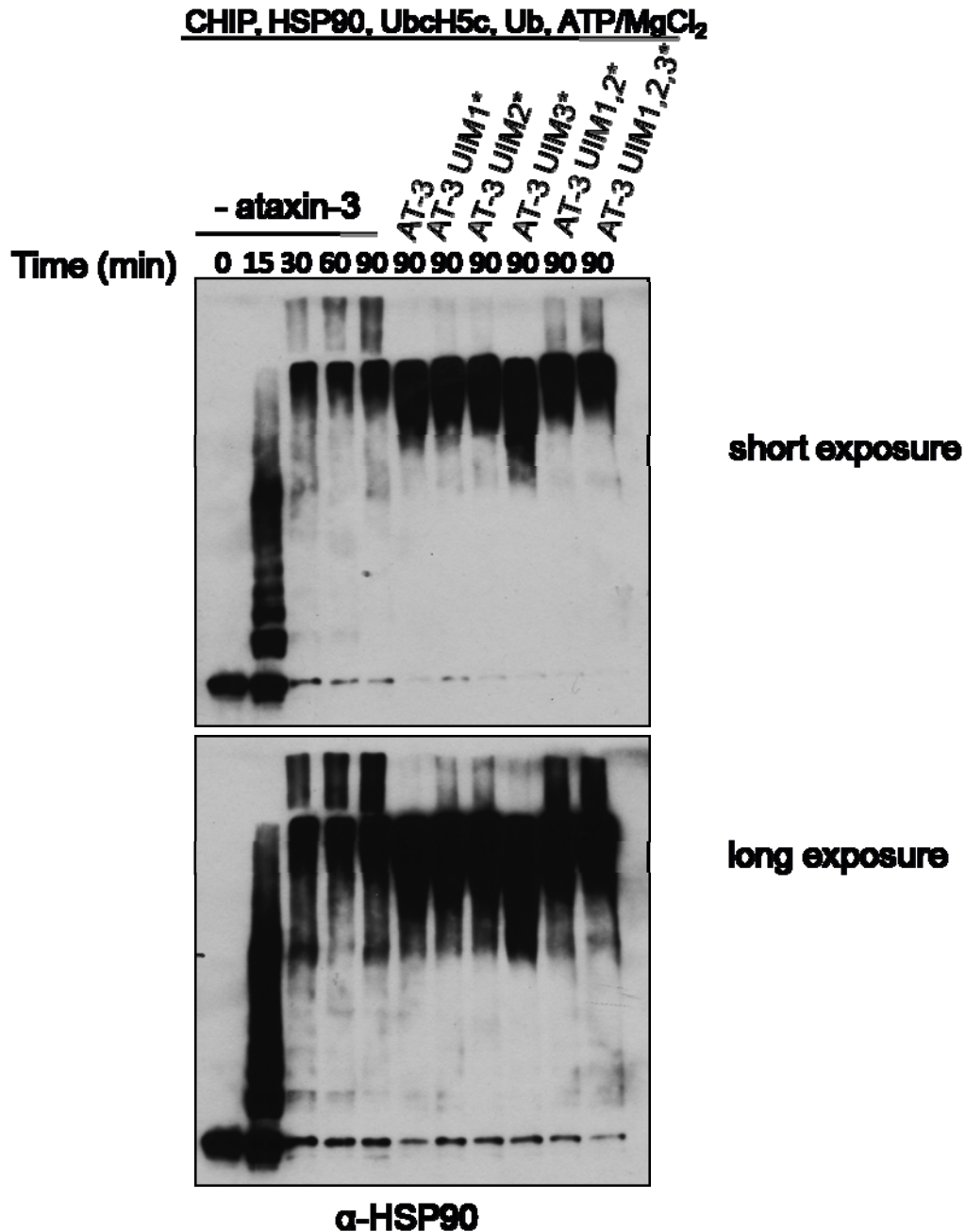
Figure 7. UIMs 1 and 2 of ataxin-3 are essential for its chain-trimming ability
Ubiquitination of Hsp90 was carried out with CHIP and UbcH5c in the presence of various forms of ataxin-3, as indicated. The stacking gel is included for the purpose of showing the presence of extremely high molecular weight species that are unable to penetrate the resolving gel. High molecular weight polyubiquitinated Hsp90 (untrimmed chains) are seen when ataxin-3 is not included, or when either its UIM 1 or 2 are mutated. In contrast, ubiquitin chains are trimmed with wild-type ataxin-3, or when only the third UIM is mutated.

Figure 8. Proposed mechanism
Misfolded substrate (curved black line) is bound by Hsp90, which is associated with the TPR domain of CHIP. Ube2w monoubiquitinates both substrate and CHIP itself. UbcH5c (Ube2d3) extends ubiquitin chains on the substrate protein. Ataxin-3 binds to the ubiquitin on CHIP via either its first or second UIM (shown in red). Its catalytic domain is shown in blue. Through mechanisms that have yet to be clarified, ataxin-3 assists in the modification of ubiquitin chains on substrate, making them ideally suited for targeting to the proteasome, where the misfolded protein substrate is consequently degraded.

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