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In vivo suppression of polyglutamine neurotoxicity by C-terminus of Hsp70 interacting protein (CHIP) supports an aggregation model of pathogenesis

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Abstract

Perturbations in neuronal protein homeostasis likely contribute to disease pathogenesis in polyglutamine (polyQ) neurodegenerative disorders. Here we provide evidence that the co-chaperone and ubiquitin ligase, CHIP (C-terminus of Hsp70-interacting protein), is a central component to the homeostatic mechanisms countering toxic polyQ proteins in the brain. Genetic reduction or elimination of CHIP accelerates disease in transgenic mice expressing polyQ-expanded ataxin-3, the disease protein in Spinocerebellar Ataxia Type 3 (SCA3). In parallel, CHIP reduction markedly increases the level of ataxin-3 microaggregates, which partition in the soluble fraction of brain lysates yet are resistant to dissociation with denaturing detergent, and which precede the appearance of inclusions. The level of microaggregates in the CNS, but not of ataxin-3 monomer, correlates with disease severity. Additional cell-based studies suggest that either of two quality control ubiquitin ligases, CHIP or E4B, can reduce steady state levels of expanded, but not wild-type, ataxin-3. Our results support an aggregation model of polyQ disease pathogenesis in which ataxin-3 microaggregates are a neurotoxic species, and suggest that enhancing CHIP activity is a possible route to therapy for SCA3 and other polyQ diseases.

Keywords

spinocerebellar ataxia; polyglutamine; microaggregate; toxic species; protein misfolding; C-terminus of Hsp70-interacting protein

Introduction

The crowded intracellular environment presents obstacles to proper folding of proteins. Accordingly, cells contain a molecular chaperone network that facilitates protein folding and promotes degradation of misfolded proteins. Its importance in the nervous system is highlighted by various neurodegenerative disorders, in which protein conformational defects cause detrimental accumulation of misfolded or aggregated protein. Protein misfolding and

aggregation may underlie neurodegeneration in diseases such as Parkinson's disease, Alzheimer's disease and the polyglutamine (polyQ) diseases (Taylor et al, 2002; Selkoe, 2004; Walsh and Selkoe, 2004). Although protein aggregation occurs in all of these diseases, how protein misfolding and aggregation damages neurons is unclear. In the polyQ diseases, moreover, it is unknown which protein species are neurotoxic: monomers, oligomers, or higher order aggregates.

Aggregation is a common byproduct of protein misfolding, suggesting that protein quality control (PQC) systems are important in countering diseases where aggregation occurs. Two PQC pathways implicated in disease are the molecular chaperone system and the ubiquitin-proteasome pathway (Bence et al, 2001; Hay et al, 2004; Marques et al, 2006). The cochaperone and ubiquitin ligase, CHIP, links these two systems. Through its N-terminus, CHIP binds heat shock proteins and modulates protein refolding (Ballinger et al, 1999; Connell et al, 2001). Through its C-terminus, CHIP ubiquitinates substrates and targets them for degradation (Connell et al, 2001; Meacham et al, 2001).

The dual roles of CHIP in chaperone- and ubiquitin-dependent pathways suggest it might be important in neuronal PQC. Numerous studies support a role for CHIP in countering neurodegenerative diseases. For example, CHIP enhances the activity of parkin, a protein associated with recessive parkinsonism (Imai et al, 2002), and regulates the degradation of phosphorylated tau, a protein implicated in various neurodegenerative diseases (Dickey et al, 2006; Dickey et al, 2007a). Although CHIP modulates the toxicity of several polyQ disease proteins (Jana et al, 2005; Miller et al, 2005; Al-Ramahi et al, 2006; Choi et al, 2007; Branco et al 2008), the precise role of CHIP in polyQ diseases is difficult to delineate because it may regulate polyQ proteins differently depending on protein context (Al-Ramahi et al, 2006; Bulone et al, 2006; Dickey et al, 2007b; Branco et al 2008).

Expansion of the polyQ domain in the ataxin-3 protein causes the polyQ disease, SCA3. While normal ataxin-3 can self-associate (Gales et al, 2005), polyQ expansion enhances its aggregation and promotes amyloid-like aggregate formation (Chow et al, 2004; Ellisdon et al, 2006). Here, we used transgenic mice expressing full-length expanded ataxin-3 (Goti et al, 2004) as a disease model of polyQ protein misfolding to: 1) define the role of CHIP in disease and 2) identify putative toxic protein species in SCA3. We find that CHIP regulates the level of expanded (pathogenic) ataxin-3 in neuronal cells, and the solubility of expanded ataxin-3 in the brain. Additionally, reduction in CHIP expression enhances expanded ataxin-3 toxicity *in vivo*. Since CHIP reduction exacerbates the disease phenotype and increases microaggregate levels in the brain, our results support an aggregation-based model of polyQ disease pathogenesis.

Methods

Mouse strains

Q71-B hemizygous mice, a mouse model of SCA3/MJD (Goti et al, 2004) were bred to *CHIP* haploinsufficient mice (Dai et al, 2003) to obtain Q71-B hemizygous transgenic/*CHIP* haploinsufficient mice ($Q^{+/-}C^{+/-}$). These F1 progeny were bred to *CHIP* haploinsufficient mice to obtain F2 generation Q71-B mice with zero, one or two *CHIP* alleles. Mice were genotyped by standard two-way PCR as previously described (Goti et al, 2004; Dai et al, 2003). Q71-B/*CHIP* mice were maintained on a mixed genetic background (C3H/HeJ/C57BL6 $\times$ 129SvEv/C57BL6). Survival analysis was performed by determining how many animals of each genotype died by 12 months of age, excluding animals that were euthanized for tissue collection at predetermined time points. This was plotted as the percentage of animals of that genotype still alive at a specific month of age.

Rotarod analysis

Mice were trained on an accelerating rotarod apparatus (Ugo Basile, Comerio, Italy) for 2 days prior to testing. Training consisted of acclimating animals to the rotarod for 2 trials per day with 10 minutes rest between trials. For testing, the apparatus was set to accelerate from 3 to 30 rpm over the course of 5 minutes. Animals performed 3 trials with at least 10 minutes rest between trials. Trials ended when animals fell off the rod, began to rotate passively (i.e. clinging to the rod as it rotated) or completed the trial.

Open field analysis

Animals were tested for spontaneous activity using the Open Field 16×16 Photobeam Activation System and Flex-Field software (San Diego Instruments, San Diego, CA). Naïve animals were placed into a 41cm × 41cm clear plastic box inside a 16×16 array of infrared beams, and movement was calculated as the number and sequence of beam breaks over the course of 30 minutes. Animals were tested under dark conditions during their dark cycle (between 18:30–22:30 hrs).

Statistical Analysis

Statistical analyses were performed using Microsoft Excel with the Data Analysis ToolPak. Behavioral data were grouped by age and genotype, then tested for significance with a two-way ANOVA for independent samples. When main effects were significant at $p < 0.05$, they were also tested by one-way ANOVA for independent samples, then significant differences were further tested post-hoc using Tukey's Honestly Significant Difference (HSD, calculated online at <http://web.mst.edu/~psyworld/virtualstat/tukeys/tukeycalc.html>).

Echocardiography

Echocardiography was performed in sedated mice as previously described (Weiss et al, 2006). Mice were sedated with midazolam, and a 15 MHz linear array probe was applied horizontally to the chest. The imaging probe was coupled to a Sonos 5500 imager (Philips Medical Systems, Bothell, WA), generating ~180–200 two-dimensional frames per second in both short- and long-axis LV planes. Images were acquired and analyzed in blinded fashion with custom-designed software (Freeland Medical Systems, Louisville, CO). Endocardial and epicardial borders were traced in the short-axis plane at end-diastole and end-systole. The lengths from left ventricular outflow tract to endocardial apex and epicardial apex, respectively, were measured at end-diastole and end-systole. The bi-plane area-length method (Hill et al, 2000) was used to calculate end-diastolic and end-systolic left ventricular volumes and ejection fraction.

Immunohistochemistry and immunofluorescence

Mice were perfused transcardially with cold sterile PBS followed by 4% paraformaldehyde in PBS. Brains were fixed overnight in 4% paraformaldehyde, rinsed in PBS overnight, then cryoprotected in 30% sucrose for at least 18 hrs at 4°C. Brains were cut serially into 30µm floating sections on a sledge microtome (Leitz) and stored at –20°C in cryoprotectant (5mM sodium phosphate pH 7.4, 75mM sodium chloride, 30% w/v sucrose, 30% v/v ethylene glycol) until use. For DAB immunohistochemistry with polyclonal antibodies, sections were blocked in H₂O₂ to quench endogenous peroxidase activity, then blocked in 5% normal goat serum and incubated in primary antibody (rabbit anti-MJD 1:1000 (Paulson et al, 1997) or rabbit anti-ubiquitin 1:500 (Dako USA, Carpinteria, CA)). Sections were rinsed and incubated in biotinylated goat-anti-rabbit 1:500 (Vector Laboratories, Burlingame, CA), then developed in 0.5mg/ml DAB (Sigma, St. Louis, MO) with 9% H₂O₂, rinsed and mounted on gelatin-coated slides. For DAB immunohistochemistry with monoclonal GFAP-Cy3 antibody (Sigma), we used the Mouse-on-Mouse Peroxidase Kit (Vector Laboratories) according to manufacturer's

instructions. To quantify ubiquitin-positive inclusions, every 24th section of the brain was stained for ubiquitin and the total number of inclusions in sections of pontine nuclei were counted. For immunofluorescence, samples were fixed in 4% paraformaldehyde, rinsed in PBS, then incubated overnight with primary antibodies in 5% normal goat serum: anti-FLAG antibody for cell culture experiments (mouse anti-FLAG M5, Sigma, 1:500) or, for brain immunofluorescence, a combination of rabbit anti-ubiquitin (Dako, 1:500) and mouse anti-ataxin-3 (1H9, provided by Y. Trottier, 1:500). The next day, samples were rinsed and incubated in fluorescent secondary antibodies (goat anti-mouse Alexa 488 with or without goat anti-rabbit Alexa 568, Molecular Probes, Carlsbad, CA, 1:500) and nuclei were counterstained with DAPI (Sigma). Cells were coverslipped and sealed with Vectashield Hard-Set fluorescence mounting medium (Vector Laboratories).

Tissue collection and lysates

Mice were perfused transcardially with 20ml cold sterile PBS plus protease inhibitor cocktail (PI) (Complete Mini tablets, Roche, Indianapolis, IN). Whole tissues were dissected, minced in RIPA (50mM Tris-HCl pH 7.4, 150mM sodium chloride, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) with PI, then homogenized in a Dounce homogenizer in RIPA plus PI lysis buffer. Lysates were diluted to 100mg/ml (based on wet weight) in RIPA plus PI lysis buffer and centrifuged at 4000 rpm for 15 minutes at 4°. The pellet was rehomogenized in the same volume of RIPA plus PI. Lysates were stored at -80° until use.

Western blot analyses

Soluble fractions were sonicated twice for 10 seconds each, then spun at 15,000×g for 10 minutes at 4°C to remove particulate debris (these pellets were not saved). Pellets from first 4000 rpm spin were vortexed and sonicated vigorously. Samples were diluted 1:1 in 2× Laemmli buffer plus DTT, then separated on discontinuous 10% or 12% acrylamide gels, or pre-cast 4–20% SDS Tris-HCl gradient gels (BioRad), and transferred to PVDF membranes. For SDS-agarose gels, gels contained 1.5% w/v high-melting-point agarose (ISC Bioexpress, Kaysville, UT) in TAE buffer (40mM Tris-acetate, 1mM EDTA) with 0.1% SDS. SDS-agarose gels were run in protein running buffer (5mM Tris base, 38mM glycine, 0.02% SDS) and transferred to PVDF membranes using a BioRad TransBlot SD semi-dry apparatus in a discontinuous buffer system (cathode buffer: 12mM Tris base, 8mM CAPS, 0.1% SDS; anode buffer: 12mM Tris base, 8mM CAPS, 15% methanol) (Bagriantsev et al, 2006). Membranes were briefly rinsed in Tween-TBS, blocked in 5% milk in Tween-TBS, and probed with the following primary antibodies: rabbit anti-human MJD 1:20,000 (Paulson et al, 1997), mouse anti-polyglutamine 1:2000 (1C2, Chemicon, Temecula, CA), mouse anti- α -GAPDH 1:500 (Chemicon), rabbit anti-CHIP 1:1000 (Calbiochem, San Diego, CA), rabbit anti-CHIP 1:1000 (abcam, Cambridge, MA), mouse anti-Hsp90 1:1000 (SPA-830, Stressgen, Ann Arbor, MI), mouse anti-Hsp70 1:1000 (SPA-810, Stressgen), rabbit anti-Hsp40 1:10,000 (Stressgen), rabbit anti-Bag-1 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Hop 1:1000 (SRA-1500, Stressgen), mouse anti-E4B 1:500 (BD Biosciences, San Jose, CA), or mouse anti- α -tubulin 1:20,000 (Sigma). Membranes were rinsed in milk and incubated in peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies 1:15,000 (Jackson ImmunoResearch, West Grove, PA) and imaged by enhanced chemiluminescence (Western Lightning, Perkin Elmer, Waltham, MA). Quantification of immunoblots was performed with NIH ImageJ software (NIH, Bethesda, MD).

Size exclusion chromatography

Soluble fractions of brain lysates were sonicated and centrifuged at 15,000×g for 10 min. 200 μ l of this lysate was loaded onto a Waters BioSuite 450 column (Milford, MA), and run in mobile phase buffer (150mM sodium chloride, 50mM sodium phosphate, pH 7.4) at 0.5ml/min.

Fractions were collected every 2 minutes, and precipitated with 14% trichloroacetic acid for 30 min on ice. Fractions were centrifuged at 15,000×g for 30 min at 4°C, pellets were washed twice with ice-cold acetone, and then were resuspended in 2× Laemmli buffer plus DTT. Fractions were analyzed by SDS-PAGE on 10% polyacrylamide gels, and processed by Western blot for ataxin-3. Column was calibrated with NativeMark protein standards (Invitrogen, Carlsbad, CA).

Denature/renature immunoprecipitation

200μl brain lysate was sonicated and centrifuged as described above and diluted with 800μl RIPA buffer. Samples were denatured with SDS (1% final concentration) for 30 minutes at room temperature, then renatured with Triton X-100 (4.5% final concentration) for 30 minutes at room temperature. Ataxin-3 was immunoprecipitated from each lysate with 4μl MJD antibody and 25μl EZview Red Protein A Affinity Gel beads (Sigma) for 4 hours at 4°C. Beads were washed 4 times with cold RIPA buffer, then protein was eluted with 60μl 2× Laemmli buffer plus DTT by boiling for 2 minutes.

Cell culture and transfection

FLP-In HEK-293 cells (Invitrogen) were engineered to stably express FLAG-tagged ataxin-3 with either 22 or 80 glutamine repeats, as described (Todi et al, 2007). FLP-In HEK-293 cells were maintained in complete FRT medium (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin, 0.2% hygromycin B). M17 human neuroblastoma cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were transfected with Lipofectamine PLUS (Invitrogen) according to the manufacturer's recommendations. The following constructs were used, alone or in combination: empty pcDNA3.1 vector, FLAG-ataxin-3-Q25, FLAG-ataxin-3-Q80, pcDNA3.1-CHIP (provided by C. Patterson, Chapel Hill, NC), and pCMV-HA-E4B (provided by K. Nakayama, Fukuoka, Japan). For steady-state ataxin-3 level experiments in M17 cells, constructs were expressed for 48 hr prior to cell harvest.

Pulse-chase analysis

FLP-In HEK-293 cells stably expressing FLAG-ataxin-3-Q22 or FLAG-ataxin-3-Q80 were transiently transfected with empty pcDNA3.1 vector, pcDNA3.1-CHIP or pCMV-HA-E4B. Forty-eight hr after transfection, cells were starved for 30 minutes in DMEM without methionine and cysteine (Gibco, Carlsbad, CA) supplemented with 2mM glutamine (Gibco). Cells were pulsed for 40 minutes in starvation medium plus ³⁵S-labeled methionine and cysteine mix (0.22μCi/μl in a 60cm dish, EasyTag EXPRESS Protein Labeling Mix, Perkin Elmer), then chased for 0, 8 or 24 hr in complete FRT medium supplemented with 2mM unlabeled methionine and cysteine. Cells were harvested from plates in PBS plus PI, lysed in RIPA plus PI, then subjected to immunoprecipitation using EZview Red ANTI-FLAG M2 Affinity agarose gel (Sigma) and subsequent elution with 3× FLAG peptide (Sigma). Immunopurified samples were run on 12% SDS-PAGE gels for autoradiography, and autoradiograms were analyzed with NIH ImageJ software.

Results

Reduction or absence of CHIP worsens polyQ disease in Q71-B transgenic mice

Because CHIP has been shown to regulate polyglutamine toxicity (Miller et al, 2005; Jana et al, 2005; Al-Ramahi et al, 2006; Choi et al, 2007), we hypothesized that reducing CHIP would exacerbate the phenotype of Q71-B mice, a cDNA transgenic model of SCA3/MJD (Goti et al, 2004). These mice overexpress human expanded, full length ataxin-3 with 71 glutamine repeats, which is within the pathologic range of glutamine expansion for SCA3 in humans.

Hemizygous Q71-B mice ($Q^{+/-}$) were crossed with *CHIP*-deficient mice to generate Q71-B mice with zero ($C^{-/-}$), one ($C^{+/-}$) or two alleles ($C^{+/+}$) of *CHIP*. Q71-B hemizygous mice with a full complement of *CHIP* ($Q^{+/-}C^{+/+}$) are phenotypically indistinguishable from wild-type mice ($Q^{-/-}C^{+/+}$) (Fig. 1), consistent with the initial report of hemizygous Q71-B mice indicating no behavioral phenotype and a normal life span (Goti et al, 2004). In contrast, hemizygous Q71-B mice lacking *CHIP* ($Q^{+/-}C^{-/-}$) develop severe, progressive neurologic deficits including kyphosis, pelvic depression, hindlimb splaying and whole body tremor, dying at 3–6 months (Fig. 1 and Fig. S1). Q71-B mice with a single *CHIP* allele ($Q^{+/-}C^{+/-}$) display an intermediate phenotype: while they do not die prematurely, they start to develop mild, progressive motor incoordination around 6 months of age and an action-dependent, body tremor by 9 months of age (Fig. 1A and Fig. S1). *CHIP*-null mice that do not express expanded ataxin-3 ($Q^{-/-}C^{-/-}$) occasionally develop kyphosis but without premature death, tremor, or other neurological signs (Fig. 1B–C).

In *CHIP*-haploinsufficient Q71-B mice ($Q^{+/-}C^{+/-}$), we searched for milder, quantifiable motor deficits. Mice were screened for motor incoordination and impaired general activity with the rotarod and open field tests, respectively (outlined in Fig. 2A). Rotarod testing revealed age-dependent motor incoordination in $Q^{+/-}C^{+/-}$ mice that became statistically significant by 9–11 months (Fig. 2B). Likewise, in open field testing, $Q^{+/-}C^{+/-}$ mice displayed significantly decreased spontaneous activity by 9 months (Fig. 2C). In contrast, Q71-B mice with normal *CHIP* levels ($Q^{+/-}C^{+/+}$) did not develop age-related motor abnormalities in either assay (Fig. 2B–C), whereas $Q^{+/-}C^{-/-}$ mice showed statistically significant motor incoordination by rotarod analysis prior to their accelerated, early death (Fig. S2). Thus, the Q71-B behavioral phenotype increases in severity as *CHIP* is reduced from two to one to zero alleles, indicating that *CHIP* suppresses polyQ disease in a dose-dependent manner.

The progressive neurobehavioral phenotype caused by expanded ataxin-3 in *CHIP*-null mice suggests that the observed early death directly reflects CNS dysfunction. But since *CHIP*-null mice are sensitive to cardiac stress (Zhang et al, 2005), we sought to exclude cardiac dysfunction as the cause of death. Transthoracic echocardiography was performed on mice from the Q71-B/*CHIP* cross. $Q^{+/-}C^{-/-}$ mice displaying behavioral symptoms exhibited normal cardiac function (Table 1) without evidence of cardiac hypertrophy, infarction, or valvular defects (data not shown). Therefore, we conclude that the early demise of $Q^{+/-}C^{-/-}$ mice reflects CNS rather than cardiac dysfunction.

CHIP reduction exacerbates neuropathological markers in Q71-B mice

In parallel with the worsened behavioral phenotype, we tested whether *CHIP* reduction also caused earlier or more severe polyQ-associated neuropathology in Q71-B mice. To do this, we examined brain sections from $Q^{+/-}C^{+/-}$ mice for the presence of intranuclear inclusions and for altered subcellular localization of ataxin-3. By six months of age, ubiquitin-positive inclusions were detected in pontine neurons of $Q^{+/-}C^{+/-}$ mice (average number of inclusions per section = 6.33, s.d. = 5.9, n = 3 animals) but not in age-matched $Q^{+/-}C^{+/+}$ animals (average number of inclusions per section = 0.00, s.d. = 0.0, n = 2 animals) (Fig. 3A). Importantly, no ubiquitin-positive inclusions were detected at 3 months of age in either genotype, although 3-month-old $Q^{+/-}C^{+/-}$ mice did display increased ubiquitin immunostaining in some pontine neurons (Fig. 3A, arrows and inset). No inclusions were detected in the hippocampus, a brain region spared in SCA3, at 3 or 6 months in either genotype (Fig. 3B). Ubiquitin-positive inclusions were not detected in other brain regions, including cerebellar cortex, deep cerebellar nuclei, striatum, thalamus, and brainstem nuclei at 3 or 6 months of age (data not shown). Because expanded ataxin-3 is expressed throughout the CNS of both $Q^{+/-}C^{+/+}$ and $Q^{+/-}C^{+/-}$ animals (Fig. 3C, Fig. S3A, and data not shown), the region-specific, age-dependent inclusion formation we observe is not attributable to regional differences in transgene expression. These

ubiquitinated inclusions contain ataxin-3, as shown by double immunofluorescence (Fig. 3D). Although such inclusions are not likely to be pathogenic (Branco et al, 2008), they do demonstrate that the neuropathology seen in Q71-B mice is similar to that observed in SCA3 patients. Expanded ataxin-3 localized primarily to neuronal cell nuclei by 3 months of age in all examined brain regions of Q71-B mice, regardless of CHIP status (Fig. 3C, Fig. S3A, and data not shown); however, ataxin-3 nuclear staining was more intense in $Q^{+/-}C^{+/-}$ mice than in $Q^{+/-}C^{+/+}$ mice at all time points. Endogenous murine ataxin-3 in nontransgenic mice, which immunostains much less robustly than expanded human ataxin-3, appears diffuse throughout the neuronal cytoplasm (Fig. S3A). Both ubiquitin and ataxin-3 immunostaining are similar in $Q^{+/-}C^{-/-}$ mice and $Q^{+/-}C^{+/-}$ mice, albeit with more complete nuclear localization and more intense staining of ataxin-3 in $Q^{+/-}C^{-/-}$ mice (Fig. S3B). In agreement with previously published results from hemizygous Q71-B mice (Goti et al, 2004), we observed no increase in the astrocytic marker, glial fibrillary acidic protein (GFAP), in affected and unaffected brain regions in Q71-B/CHIP mice (Fig. S4), suggesting that there is not significant astrogliosis.

CHIP reduction leads to increased ataxin-3 microaggregates in Q71-B mouse brain

The dose-dependent phenotype caused by CHIP reduction in Q71-B mice allowed us to determine whether CHIP reduction enhances ataxin-3 aggregation and to correlate biochemically detectable changes in aggregation to behavioral phenotype. CHIP has been shown to promote the degradation of polyQ proteins and/or modulate their aggregation (Choi et al, 2007; Al-Ramahi et al, 2006; Miller et al, 2005; He et al, 2004). Consistent with this, reducing or eliminating CHIP in Q71-B mice resulted in a marked, stepwise increase in expanded ataxin-3 species that electrophorese on SDS-PAGE as high molecular weight complexes after sonication and centrifugation to remove particulate matter (Fig. 4A, bracket). Because these ataxin-3 complexes are detectable at 3 months, when few if any inclusions are present, and are resistant to denaturing detergent yet partition into the soluble fraction of brain lysates, we conclude that they represent detergent-resistant microaggregates. Very few microaggregates partition in the brain pellet (Fig. S5). While these microaggregates may be pure ataxin-3 oligomers, we cannot currently rule out the presence of other proteins. In contrast, CHIP reduction or elimination did not alter the levels of endogenous ataxin-3 or of monomeric, expanded (i.e. human mutant) ataxin-3 (Fig. 4A, 4B, and Fig. S6), nor did reducing CHIP levels induce formation of microaggregates from endogenous ataxin-3.

To confirm that the high-molecular-weight ataxin-3 complexes in the soluble brain fraction correspond to microaggregates rather than inclusions, we electrophoresed brain lysates on 1.5% SDS-agarose gels which, due to their large pore size, can resolve larger protein complexes than a standard polyacrylamide gel (Bagriantsev et al, 2006). The calculated pore size we used (~130nm, according to the method of Xiong et al, 2005) is too small to permit entry of inclusions into the gel. As shown in Figure 4C, CHIP reduction or elimination leads to a stepwise increase in soluble ataxin-3 microaggregates in the brain. We also tested whether the microaggregates were 1C2-positive, since this polyQ-specific monoclonal antibody is thought to detect specific, nontoxic types of polyQ oligomers (Behrends et al, 2006). As shown in Figure 4A, 1C2 detects expanded ataxin-3 monomer, but not endogenous ataxin-3 monomer or ataxin-3 microaggregates (Figs. 4A and 4C); this result is similar to 1C2 labeling of expanded huntingtin monomer versus putative toxic huntingtin oligomers (Behrends et al, 2006). Since we cannot reliably size microaggregates on SDS-agarose gels (Fig. S7), we performed size exclusion chromatography on clarified brain lysates to assess microaggregate size. Microaggregates from $Q^{+/-}C^{+/+}$ mouse brain fractionated between ~700–7000kD in size, confirming that they are soluble high-molecular-weight complexes (Fig. 5). Reduction or loss of CHIP corresponded with progressively greater levels of microaggregates compared to monomeric ataxin-3 species, but did not change the fractions in which microaggregates or monomeric ataxin-3 eluted (Fig. 5). Stringent immunoprecipitations employing a denature-renature step further demonstrated

that ataxin-3 microaggregates are not highly ubiquitinated (Fig. S8), arguing that the large electrophoretic shift in these HMW ataxin-3 species is not due to conjugation to poly-ubiquitin chains. Although the Q71-B transgene is expressed in many tissues, ataxin-3 microaggregates were only present in the brain, not other tissues (Fig. S9). Taken together, these results indicate that 1) ataxin-3 microaggregate burden correlates with disease severity, and 2) CHIP regulates microaggregate levels in the brain *in vivo*.

Analysis of the effects of CHIP reduction on specific heat shock protein levels in brain

CHIP activates HSF1 and triggers expression of heat shock proteins (Dai et al, 2003), and loss of CHIP has been reported to correlate with a decrease in Hsp90 and Hsp40 levels in mouse brain (Dickey et al, 2007a). CHIP also induces Hsp70 expression during conditions of increased proteotoxic stress, and triggers Hsp70 degradation once cells return to basal conditions (Qian et al, 2006). Some studies have shown that when mutant polyQ protein is present, levels of stress-associated proteins, including some heat shock proteins, are dysregulated (Tagawa et al, 2007; Hay et al, 2004). Perhaps the simplest explanation for the worsened disease phenotype when CHIP is reduced or eliminated would be a deleterious imbalance in molecular chaperones. Therefore, we asked whether Q71-B mice with reduced or absent CHIP have lower levels of key heat shock proteins compared to Q71-B mice with normal CHIP levels.

Expression of expanded ataxin-3 on all CHIP backgrounds does not increase Hsp90 or Hsp40 above wild-type levels (Fig. 6), suggesting no major imbalance in these key chaperones. Hsp70 levels trended higher in CHIP-null animals with or without expanded ataxin-3 (Fig. 6B), consistent with results reported in Qian et al. (2006), but did not reach statistical significance (Table S1). Removal of one Q^{-/-}C^{-/-} animal from the analysis, thereby reducing the variability of the results, revealed that CHIP-null animals express significantly higher levels of Hsp70 than wild-type animals (Fig. S10), but this effect is independent of expanded ataxin-3 expression (Table S2). Neither levels of Bag1 nor of Hop (co-chaperones for CHIP and Hsp90, respectively) varied with CHIP levels (Fig. 6A). Additionally, the levels of E4B, another protein quality control ubiquitin ligase implicated in ataxin-3 regulation, did not change with expression of expanded ataxin-3 or reduction in CHIP (Fig. 6A). Therefore, in this model system, CHIP reduction does not impair expression of key heat shock proteins when polyQ disease protein is present. We conclude that CHIP reduction does not enhance polyQ toxicity primarily by altering levels of these key molecular chaperones.

Two protein quality control ubiquitin ligases can regulate levels of expanded ataxin-3 in cell culture

Whether CHIP is the primary mediator of ubiquitin-dependent degradation of ataxin-3 is controversial: Jana et al (2005) reported that CHIP ubiquitinates ataxin-3 and directs it to the proteasome for degradation, whereas Matsumoto et al (2004) reported that another ubiquitin ligase, E4B, mediates ataxin-3 degradation while CHIP has no effect. CHIP and E4B share properties indicating that both may represent protein quality control ubiquitin ligases that preferentially act on nonnative polypeptides and facilitate other PQC components (Qian et al, 2006; Richly et al, 2005). Our results above show that modulating the levels of quality control ubiquitin ligases like CHIP does influence ataxin-3 levels, but whether this is a direct or indirect effect cannot easily be assessed *in vivo*. To clarify the role of CHIP and E4B in ataxin-3 degradation, we assessed their effect on steady-state ataxin-3 levels in transiently transfected neural cells. When CHIP is overexpressed in M17 cells, steady-state levels of coexpressed nonpathogenic ataxin-3 levels remain unchanged, but pathogenic (expanded) ataxin-3 levels decrease (Fig. 7A). Similarly, overexpression of E4B selectively decreases expanded ataxin-3 levels without affecting levels of wild-type, nonpathogenic ataxin-3 (Fig. 7A). Thus, both ligases may selectively enhance the clearance of expanded ataxin-3.

In an effort to confirm these results, we performed pulse-chase assays of ataxin-3 degradation. FLP-In HEK-293 cells that stably express FLAG-ataxin-3-Q22 or FLAG-ataxin-3-Q80 were transfected with constructs expressing CHIP, E4B or an empty vector. Cells were pulse-labeled with ³⁵S-methionine/cysteine, then chased for 0, 8 or 24 hrs. FLAG-ataxin-3 was immunoprecipitated from cell lysates, then analyzed by SDS-PAGE and autoradiography. In this experimental system, CHIP did not significantly accelerate degradation of either wild-type or expanded ataxin-3 compared to empty vector (Fig. 7B, Fig. S11; Table S3). E4B may have slightly enhanced the degradation of expanded ataxin-3 but this did not reach statistical significance; E4B overexpression had no effect on wild-type ataxin-3 (Fig. 7B, Fig. S11; Table S3). It is important to note that we did not detect ataxin-3 aggregation in either of these experiments, either by immunofluorescence or western blot (Fig. S12), so these systems do not replicate all of the disease protein features observed *in vivo*.

Discussion

Our results establish CHIP as a central component of homeostasis mechanisms countering polyQ toxicity in the brain. Complete loss of CHIP converts an asymptomatic mouse model of SCA3, the hemizygous Q71-B mouse, into one with an early onset, rapidly progressive behavioral phenotype with massive accumulation of soluble ataxin-3 microaggregates (Fig. 1, Fig. 4, Fig. 5, Fig. S1, S2, S5). CHIP haploinsufficiency in the same model causes a less severe phenotype with moderate levels of ataxin-3 microaggregates (Fig. 1, Fig. 2, Fig. 4, Fig. 5). The fact that we observe robust phenotypic exacerbation with loss of a single *CHIP* allele highlights the importance of CHIP in decreasing expanded ataxin-3 toxicity. Previously we showed that CHIP haploinsufficiency exacerbates neurotoxicity in another polyQ disease, Huntington's disease (HD, Miller et al, 2005), but did not measure changes in aggregation. The dose-dependent effect of CHIP on both the progressive behavioral phenotype and microaggregate accumulation in Q71-B mice supports a causal link between microaggregates and polyQ neurotoxicity.

Although monomeric ataxin-3 levels in the brain remain unchanged when CHIP is reduced or absent (Fig. 4B and Fig. S6), ataxin-3 microaggregates accumulate. We term these complexes "microaggregates" because they partition in the soluble fraction of brain lysates and can be sized by size exclusion chromatography (Fig. 5), yet are resistant to denaturing detergents. Moreover, they accumulate before intranuclear inclusions are observed, and are distinguishable from inclusions by size. Ataxin-3 microaggregates are similar to soluble huntingtin aggregates recently identified in mouse models of HD (Weiss et al, 2008), and may represent ataxin-3 homo-oligomers or heteromeric complexes. Microaggregate levels directly correlate with neuropathological severity and behavioral disturbance in Q71-B mice, increasing stepwise as CHIP levels are reduced, while monomeric ataxin-3 levels do not. Therefore, we suggest that ataxin-3 microaggregates constitute a neurotoxic species in SCA3. We cannot, however, prove that intracellular ataxin-3 microaggregates are directly toxic *in vivo* because, unlike the case with transmissible prions, it is not possible to introduce polyQ microaggregates isolated from one animal into the neurons of another animal to establish causality.

In other neurodegenerative diseases CHIP has been shown to regulate disease proteins including α -synuclein, parkin and tau (Shin et al, 2005; Imai et al, 2002; Dickey et al, 2006 and 2007a; Tetzlaff et al, 2008). When mutated these proteins tend to misfold, promoting abnormal oligomerization. Oligomers of α -synuclein and A-beta have been implicated as neurotoxic species (Danzer et al, 2007; Lesne et al, 2006). Our results likewise support a toxic oligomer model of polyQ disease pathogenesis, with CHIP functioning to reduce levels of these complexes. In this model (Fig. 8), expanded polyQ proteins have an increased probability of misfolding (Chen et al, 2002; Bhattacharyya et al, 2005; Colby et al, 2006). Misfolded monomers may be targeted for degradation by quality control ubiquitin ligases such as CHIP

and E4B, or undergo chaperone-mediated refolding in a CHIP-facilitated manner. Misfolded polyQ monomers are prone to aggregate, forming homo-oligomers or heteroprotein complexes that may be inefficient proteasomal substrates (Bence et al, 2001; Bennett et al, 2005; Verhoef et al, 2002). Through its actions on misfolded proteins and chaperone regulation, CHIP would be expected to inhibit aggregate formation and facilitate the dissociation of aggregates.

Several recent studies have explored the identity of neurotoxic protein species in polyQ disease. For example, Behrends et al (2006) found that expanded huntingtin formed soluble oligomers that inhibited cell growth and were amyloid-antibody positive. Nagai et al (2007) showed that both monomeric and oligomeric polyQ proteins are toxic when microinjected into cells. Takahashi et al (2008) used a FRET-based assay to show that soluble polyQ oligomer formation correlated with death in neuronal cells. Our data with a full length disease protein provide animal model support for the hypothesis that soluble oligomers are the neurotoxic species in polyQ diseases. Determining whether ataxin-3 microaggregates are pure homo-oligomers or heteroprotein complexes would require that microaggregates be purified and analyzed by mass spectrometry. Their stability on agarose gels suggests this may be possible.

CHIP has been called a central regulator of HSP expression because it activates HSF-1 (Dai et al, 2003) and helps terminate the stress response by targeting Hsp70 for degradation (Qian et al, 2006). Since HSF-1 is the principal stress-induced transcription factor (Morimoto, 1998), CHIP reduction would be expected to lead to reduced levels of HSF-regulated genes (e.g. heat shock proteins). While previous studies have shown decreases in Hsp40, Hsp90 and Hop in *CHIP*-null mouse brain (Dickey et al, 2007a), we did not observe this (Fig. 6). This discrepancy could be explained by two factors: 1) our Q71-B/*CHIP* mouse line is maintained on a different background than the original *CHIP*-null line (C3H/HeJ/C57BL6 $\times$ 129SvEv/C57BL6 versus 129SvEv/C57BL6, respectively); and 2) previous studies in *CHIP*-null mice focused on a subset of “symptomatic” mice that die by 25–35 days postpartum (Dickey et al, 2006; Dickey et al, 2007a). “Symptomatic” *CHIP*-null mice constitute ~20% of the *CHIP*-null animals in a colony, while the remaining 80% of *CHIP*-null mice have a normal lifespan and remain phenotypically normal if unstressed (Dai et al, 2003). Our studies were performed in *CHIP*-null mice that survive well past 30 days. Although a recent paper shows a 50% mortality rate in *CHIP*-null mice by 12 months (Min et al, 2008), we did not observe increased mortality in our experiments unless the Q71-B transgene was also present (Fig. 1C). When CHIP is absent in our experiments, the normal expression level of the tested chaperones suggests that other neuronal pathways may activate HSF-1 or otherwise trigger chaperone gene expression. Indeed recent data show that polyQ protein expression induces Hsp70 in certain neurons in an HSF-1-independent manner (Tagawa et al, 2007). At least in the case of Hsp70, HSF-1 apparently is not the only regulator.

It should be emphasized that we only assessed certain key chaperones implicated in neurodegeneration: Hsp40, 70 and 90. We did not find significant changes in either Hsp40 or Hsp90, implying that the mechanism by which CHIP reduction worsens disease is not primarily a perturbation in the balance of these key chaperones. We found that loss of CHIP correlates with increased Hsp70 levels in most *CHIP*-null mice, independent of expanded ataxin-3 overexpression (Fig. 6B, Fig. S10; Table S1–Table S2), confirming that a) CHIP plays an important role in Hsp70 regulation, and b) changes in Hsp70 levels caused by loss of CHIP are unrelated to behavioral phenotype. Our results do not rule out CHIP-mediated effects on other undefined chaperones or accessory factors in the CNS. The CNS “chaperome” is likely vast and remains mostly uncharted. It would be useful to transcriptionally profile the functional chaperome of the mouse brain and determine which chaperone components are regulated by CHIP, both under basal and disease conditions.

CHIP acts in several ways on substrate proteins: it can refold proteins in concert with interacting chaperones (Kampinga et al, 2003; Rosser et al, 2007), ubiquitinate proteins (Marques et al, 2006), or facilitate other ubiquitin ligases (Imai et al, 2002). With respect to specific polyQ proteins, CHIP can regulate degradation of ataxin-1 and androgen receptor (Al-Ramahi et al, 2006; He et al, 2004). CHIP was also shown to regulate degradation of huntingtin (Jana et al, 2005), while another study showed that CHIP promoted polyQ-expanded huntingtin solubility rather than degradation (Miller et al, 2005). The results for ataxin-3 have been equivocal, as two groups reported opposing effects of CHIP on ataxin-3 degradation (Matsumoto et al, 2004; Jana et al, 2005). The massive accumulation of ataxin-3 microaggregates in $Q^{+/-}C^{-/-}$ mice, despite stable levels of monomeric ataxin-3, could be explained either by a failure to promote degradation when CHIP is missing, or a failure to keep ataxin-3 in a soluble, degradable state. CHIP may be important for both expanded ataxin-3 solubility and degradation. While CHIP reduction also increases inclusion formation at later times, these may be byproducts of abnormal protein accumulation rather than directly pathogenic structures. Recent work shows that CHIP-mediated changes in inclusion formation can be dissociated from its ability to suppress polyQ toxicity (Branco et al, 2008).

The regulation of ataxin-3 may be complex and involve multiple degradation pathways (Matsumoto et al, 2004; Jana et al, 2005; Todi et al, 2007). Our steady-state results show that both CHIP and E4B overexpression reduce expanded ataxin-3 levels, though neither reduces wild-type ataxin-3 levels in neuronal cells (Fig. 7A). CHIP and E4B have been shown to work together to ubiquitinate and regulate the levels of the *C. elegans* protein, UNC-45 (Hoppe et al, 2004); a similar phenomenon could underlie ataxin-3 regulation in the mammalian brain. It would be interesting to assess the effect of E4B on polyQ disease phenotype and ataxin-3 stability using the E4B-null mouse (Kaneko-Oshikawa et al, 2005). We suggest that loss of E4B, like loss of CHIP, would cause a buildup of expanded ataxin-3.

It is still unclear which processes favor aggregation of expanded ataxin-3 selectively in the brain and not elsewhere (Fig. S9). Comparative transcriptional profiling of the basal and stress-induced components of PQC in the CNS versus non-neuronal tissues might shed light on the selective vulnerability of the brain and suggest therapeutic targets. Certain classes of drugs, such as the Hsp90 inhibitors, show therapeutic promise by enhancing neuronal chaperone activity, through either increased refolding activity or targeting of chaperone client proteins for degradation (Waza et al, 2005; Thomas et al, 2006; Dickey et al, 2007a). Since CHIP is an important regulator of cellular responses to misfolded protein, enhancers of CHIP's ligase and/or co-chaperone functions might augment pathways necessary to counter polyQ disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Loss of CHIP exacerbates expanded ataxin-3 toxicity *in vivo*

A. Timeline of symptom onset for Q71-B hemizygous mice on a *CHIP* haploinsufficient ($Q^{+/-}C^{+/-}$) or null ($Q^{+/-}C^{-/-}$) background. No behavioral abnormalities developed in Q71-B mice on a *CHIP* wild-type background over the 15-month study period ($Q^{+/-}C^{+/+}$, not shown).

B. Representative photographs of 4-month-old mice from Q71-B/*CHIP* crosses. Q71-B mice lacking *CHIP* ($Q^{+/-}C^{-/-}$) develop kyphosis (arrow) and splaying of hindlimbs (arrowhead). In contrast, Q71-B mice haploinsufficient for *CHIP* ($Q^{+/-}C^{+/-}$) or wild-type for *CHIP* ($Q^{+/-}C^{+/+}$) appear healthy, as do nontransgenic mice with reduced *CHIP* ($Q^{-/-}C^{+/-}$).

Nontransgenic *CHIP* knockout mice ($Q^{-/-}C^{-/-}$) occasionally develop kyphosis, as shown here, but retain normal motor behavior and lifespan. **C.** Survival curves for Q71-B/*CHIP* crosses. Q71-B mice lacking *CHIP* ($Q^{+/-}C^{-/-}$, open circles, blue line) die by 6 months of age while all other genotypes show normal survival rates. The number of animals followed for each genotype was: $Q^{-/-}C^{+/+}$ $n=34$, $Q^{-/-}C^{+/-}$ $n=65$, $Q^{-/-}C^{-/-}$ $n=26$, $Q^{+/-}C^{+/+}$ $n=25$, $Q^{+/-}C^{+/-}$ $n=44$, $Q^{+/-}C^{-/-}$ $n=17$.

Figure 2. Reduction of CHIP causes progressive motor deficits in Q71-B mice

A. Timeline for behavioral testing and tissue collection from Q71-B/CHIP cross. Animals were tested for motor function by rotarod (arrows) and open field analyses (gray bars) at the indicated ages (top). Brains were harvested for immunohistochemistry (IHC) and western blot (WB) at the ages noted (bottom). **B.** Motor coordination was assessed by rotarod for the indicated age groups and genotypes. By 9 months of age, Q^{+/-}C^{+/-} mice (light gray) develop statistically significant motor impairment on the rotarod, measured by decreased average latency to fall from the rod compared to all younger genotypes and ages (*p<0.01, one-way ANOVA with Tukey's HSD post-hoc test; number of animals per genotype varied from 5–9 per bin.) **C.** Spontaneous motor activity was assessed by open field activity. Spontaneous movement is

measured as the total number of beam breaks in 30 minutes. By 9 months, $Q^{+/-}C^{+/-}$ mice exhibit significantly decreased spontaneous motor activity compared to all younger genotypes and ages ($\dagger p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc test; number of mice per genotype and age varied from 2–8 per bin). Data in *B* and *C* are displayed as the group mean plus standard deviation.

Figure 3. Reduction of CHIP increases age-dependent inclusion formation in the brainstem of Q71-B mice

A. Brain sections from 3- and 6-month-old mice labeled with anti-ubiquitin antibody reveal the presence of ubiquitin-positive inclusions in pontine neurons only in 6-month-old $Q^{+/-}C^{+/-}$ mice (arrowheads and inset). By 3 months of age, some pontine neurons in $Q^{+/-}C^{+/-}$ mice show increased ubiquitin immunostaining (arrows and inset), but no inclusions.

B. Brain sections from 3- and 6-month-old mice stained as in A show that no inclusions form in the hippocampus, a brain region spared in SCA3.

C. Immunolabeling with an ataxin-3 antibody reveals that the ataxin-3 transgene in Q71-B mice is expressed widely in the CNS, including in the hippocampus and pons. By 6 months of age, expanded ataxin-3 largely localizes diffusely to neuronal cell nuclei in $Q^{+/-}C^{+/+}$ and $Q^{+/-}C^{+/-}$ mice, both in typically affected regions (e.g. pons) and unaffected brain regions (e.g. hippocampus). Substantial enrichment of ataxin-3 throughout the nucleus may obscure detection of ataxin-3-positive

nuclear inclusions by this method. *D*. Double immunofluorescence labeling in $Q^{+/-}C^{+/-}$ pontine neurons shows that ubiquitin-positive (red) nuclear inclusions contain ataxin-3 (green), as shown by the yellow fluorescence in the merged image (far right). Scale bars for A, B and C = 100 micrometers, scalebar for D = 10 micrometers.

Figure 4. Reduction of CHIP increases ataxin-3 microaggregates in brain

A. Soluble fractions of brain lysates from 4.5-month-old mice were resolved on a 4–20% Tris-HCl gradient gel, then probed with the indicated antibodies. *CHIP* haploinsufficient Q71-B mice ($Q^{+/-}C^{+/-}$) develop high-molecular-weight (HMW) SDS-resistant ataxin-3 aggregates (bracket) that increase further when CHIP is absent ($Q^{+/-}C^{-/-}$ mice). Levels of soluble ataxin-3 monomer do not change significantly (arrow: endogenous murine ataxin-3; arrowhead: transgenic expanded ataxin-3). The band at ~150kD (asterisk) is nonspecific. The 1C2 anti-polyglutamine monoclonal antibody detects expanded ataxin-3 monomer, but not endogenous ataxin-3 monomer or HMW ataxin-3. GAPDH is shown as loading control. **B.** Quantification of endogenous monomeric ataxin-3 in mouse brain lysates. There is no significant difference in endogenous monomeric ataxin-3 levels between genotypes (one-way ANOVA, $\alpha=0.05$). Blot signals (shown in Fig. S6B) were quantified with ImageJ, then the mean optical density for each genotype was normalized to GAPDH and plotted. Number of brains varied from 2–5 per genotype. Error bars = 1 S.D. **C.** Brain lysates prepared as in A, but separated instead on 1.5% SDS-agarose gels, reveal ataxin-3 microaggregates that increase stepwise as CHIP is reduced or eliminated (low levels in $Q^{+/-}C^{+/+}$ mice, intermediate in $Q^{+/-}C^{+/-}$ mice, and

abundant in Q^{+/-}C^{-/-} mice). Ataxin-3 microaggregates are not recognized by 1C2 monoclonal antibody.

Figure 5. Size exclusion chromatography reveals that ataxin-3 microaggregates are soluble HMW complexes

Brain lysates from 4.5-month-old $Q^{+/-}C^{+/+}$, $Q^{+/-}C^{+/-}$ and $Q^{+/-}C^{-/-}$ mice were subjected to size exclusion chromatography, and fractions were analyzed by SDS-PAGE and Western blot for ataxin-3. Soluble ataxin-3 microaggregates elute after the void in fractions 4 and 5, which contain complexes ranging from ~700–7000kD in size. When CHIP is reduced or absent, ataxin-3 microaggregate levels increase, especially in fraction 4. The sizing range of the column is 20–7000kD. Bracket: HMW ataxin-3 microaggregates; arrowhead: transgenic expanded ataxin-3; arrow: endogenous murine ataxin-3. The band at ~150kD (asterisk) is nonspecific.

Figure 6. CHIP loss does not significantly alter specific stress-response protein levels

A. In Q71-B mice, Hsp90 and Hsp40 levels are not greatly increased upon CHIP reduction or removal (compare lane 3, 4, 5). Bag1, Hop, and E4B levels are not altered in any genotype. **B.** Further quantification of heat shock protein levels in mouse brain lysates. Levels of Hsp90 and Hsp40 do not significantly differ between wild-type (Q^{-/-}C^{+/+}), CHIP knockout (Q^{-/-}C^{-/-}), and Q^{+/-}C^{-/-} mice (one-way ANOVA, alpha=0.05). While Hsp70 levels trend higher in Q^{-/-}C^{-/-} and Q^{+/-}C^{-/-} animals compared to wild-type animals, consistent with the fact that CHIP regulates Hsp70 levels (Qian et al, 2006), this does not reach statistical significance (one-way ANOVA, alpha=0.05, shown in Table S1). Blot signals (top) were quantified with ImageJ, then the mean optical density for each genotype was normalized to

GAPDH and plotted as a fraction of the wild-type ($Q^{-/-}C^{+/+}$) signal (bottom). Error bars = 1 S.D.

Figure 7. Analysis of the role of CHIP and E4B in regulating levels of expanded ataxin-3

A. Both CHIP and E4B reduce steady state levels of expanded, but not wild-type, ataxin-3. M17 neuroblastoma cells were transiently cotransfected with the indicated plasmids, and lysates were then examined by western blot. Representative blots are shown, with the proteins detected by specific antibodies shown at left. Ataxin-3 signal was quantified using NIH ImageJ, normalized to GAPDH loading control, then plotted at right as a fraction of the signal from the empty vector control condition. Shown are means (3 independent trials) plus S.D. CHIP or E4B expression significantly reduced expanded ataxin-3 levels (* $p < 0.01$ by Student's t -test, comparison to results with empty vector control). **B.** Effect of CHIP and E4B on degradation of normal or expanded ataxin-3. 48 hrs after transfection with the indicated plasmids, FLP-In HEK293 cells stably expressing FLAG-ataxin-3-Q22 or FLAG-ataxin-3-Q80 were pulse labeled with ^{35}S -labeled methionine/cysteine and chased for the indicated times. Plotted are the ataxin-3 signals detected by autoradiography and normalized to ataxin-3 signal immediately after pulse labeling (means of 3 independent experiments). Neither CHIP (gray line) nor E4B (dotted line) significantly enhanced clearance of normal or expanded ataxin-3, though E4B displays a trend toward enhancing expanded ataxin-3 degradation (one-way ANOVA, $\alpha = 0.05$). Representative autoradiograms of each experiment are shown above the graphs.

Figure 8. Model of CHIP action on mutant ataxin-3 in brain

This model depicting cellular pathways in the presence (A) and absence (B) of CHIP is based both on results reported here and from previously published studies (Matsumoto et al, 2004; Jana et al, 2005; Miller et al, 2005). Thick arrows indicate predominant pathways, thin arrows less predominant pathways, and dotted arrows unfavored pathways in the given scenario. A. Expanded ataxin-3 monomer (A3) (1) has an increased probability of misfolding (2) and forming aberrant heteroprotein and homo-oligomeric complexes (8 and 9, respectively). Misfolded ataxin-3 monomer can be ubiquitinated by E4B (3) or CHIP (6) and targeted for proteasomal degradation (steps 4–5). Misfolded ataxin-3 monomers or oligomers may also bind to CHIP and undergo chaperone-mediated refolding (7). Adequate levels of CHIP favor

dissociation and clearance of misfolded aggregated ataxin-3. *B.* When CHIP is reduced or absent, handling of expanded ataxin-3 is impaired. Consequently ataxin-3 microaggregates, comprising hetero-protein complexes (8) and/or homo-oligomers (9), accumulate with deleterious consequences for the neuron. Such microaggregates may represent relatively poor substrates for the proteasome and other ataxin-3 clearance pathways.

Table 1**Q71-B mice lacking CHIP have normal cardiac function**

Male and female Q^{+/-}C^{-/-} mice were assessed by transthoracic echocardiography. Each row in the table represents one mouse. Although there is no standard in the literature for the normal EF in this mixed background mouse line, previous studies report an EF of 80% +/- 5% in adult C57BL/6 mice (Weiss et al, 2006).

Q71-B	CHIP	Sex	Age (wks)	EF
-/-	+/+	M	11	78.82
+/-	+/-	M	11	83.80
+/-	-/-	M	11	74.68
-/-	+/+	F	16	74.91
-/-	-/-	F	16	83.06
+/-	+/-	F	16	70.99
+/-	-/-	F	16	81.09