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The Deacetylase HDAC6 Regulates Aggresome Formation and Cell Viability in Response to Misfolded Protein Stress

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Summary

The efficient clearance of cytotoxic misfolded protein aggregates is critical for cell survival. Misfolded protein aggregates are transported and removed from the cytoplasm by dynein motors via the microtubule network to a novel organelle termed the aggresome where they are processed. However, the means by which dynein motors recognize misfolded protein cargo, and the cellular factors that regulate aggresome formation, remain unknown. We have discovered that HDAC6, a microtubule-associated deacetylase, is a component of the aggresome. We demonstrate that HDAC6 has the capacity to bind both polyubiquitinated misfolded proteins and dynein motors, thereby acting to recruit misfolded protein cargo to dynein motors for transport to aggresomes. Indeed, cells deficient in HDAC6 fail to clear misfolded protein aggregates from the cytoplasm, cannot form aggresomes properly, and are hypersensitive to the accumulation of misfolded proteins. These findings identify HDAC6 as a crucial player in the cellular management of misfolded protein-induced stress.

Introduction

Histone deacetylases (HDACs) are a family of enzymes whose functions have been overwhelmingly associated with gene expression and chromatin dynamics (reviewed in Ayer, 1999). However, recent evidence indicates that not all functions of HDACs are dedicated to regulating gene transcription and chromatin remodeling (reviewed in Verdin et al., 2003). The prominent example of such a noncanonical function is illustrated by the cytoplasmic deacetylase HDAC6. We and others have previously reported that HDAC6 is a microtubule-associated deacetylase that can regulate microtubule acetylation and chemotactic cell motility (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003; Haggarty et al., 2003). There are, however, two unusual properties associated with HDAC6 whose significance is not understood. First, HDAC6 shows extensive colocalization with $p150^{glued}$ (Hubbert et al., 2002), a component of the dynein motor complex. Second, HDAC6 contains an ubiquitin binding zinc finger, and becomes associated with ubiquitinated proteins upon inhibition of proteasome activity (Hook et al., 2002; Seigneurin-Berny et al., 2001; J.J. Kovacs and T.-P. Yao, submitted). Although it is not apparent why a deacetylase would associate with a microtubule-associated motor and ubiquitinated proteins, these observations suggest an intriguing possibility that HDAC6 might be involved in connecting dynein motor- and ubiquitin-dependent biological processes.

One critical process known to involve both dynein motors and protein ubiquitination is the management of misfolded protein aggregates. Misfolded proteins resulting from genetic mutations, inappropriate protein assembly, aberrant modifications, and environmental stress are the inevitable byproducts of biogenesis. More than simply being nonfunctional, misfolded proteins are prone to forming aggregates that can interfere with normal cellular function (Plemper and Wolf, 1999). Thus, misfolded proteins are closely monitored, processed, and degraded to prevent their accumulation in cells. The degradation of misfolded proteins, which are recognized and often polyubiquitinated by a complex network of proteins, is carried out primarily by the proteasome (reviewed in Kopito, 1997). However, once aggregated, misfolded proteins are not degraded efficiently by proteasome machinery. As aggregated proteins are toxic, their efficient disposal is essential for cell survival (reviewed in Kopito, 2000). In fact, failure to degrade misfolded and aggregated proteins is a dominant contributing factor to neuronal cell death in many neurodegenerative diseases (Thomas et al., 1995; Lam et al., 2000).

The pathway responsible for the clearance of misfolded protein aggregates is poorly understood. The recent discovery of a novel cellular structure, the aggresome, provides an important clue that may help elucidate the players in this pathway. Aggresomes were first identified in the characterization of a mutant form of the cystic fibrosis transmembrane conducting regulator CFTR-ΔF508, which is prone to misfolding and aggregation. Similar to many misfolded proteins, misfolded CFTR-ΔF508 becomes polyubiquitinated and is degraded by proteasomes (Jensen et al., 1995; Ward et al., 1995). Upon inhibition of proteasome activity, however, misfolded CFTR-ΔF508 accumulates, forms aggregates, and strikingly, becomes concentrated into a single prominent juxtanuclear inclusion body that was termed the aggresome (Johnston et al., 1998). Subsequent studies established that aggresomes are induced by ectopic expression of many different misfolded proteins and are major repositories for misfolded protein aggregates. It has thus been proposed that aggresome formation is a specific and active cellular response to cope with excessive levels of misfolded and aggregated proteins (reviewed in Kopito, 2000). Supporting the role of aggresomes in processing misfolded protein aggregates, components of proteasomes and molecular chaperones are actively recruited to, and are abundantly present in, aggresomes (reviewed in Garcia-Mata et al., 2002; Kopito, 2000). Aggresomes are also of clinical interest as they are similar to the cytoplasmic inclusion bodies commonly observed in many neurodegenerative diseases. Aggresomes and Lewy bodies, the hallmark

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cytoplasmic inclusion bodies found in neurons affected by Parkinson's disease, share remarkable biochemical and morphological characteristics (McNaught et al., 2002). Aggresomes might therefore be fundamentally important in both misfolded protein-induced stress response and the pathogenesis of neurodegenerative disease. However, little is known about the regulation of aggresome formation.

Aggresomes are uniquely located in close proximity to the microtubule-organizing center (MTOC). This localization reflects the fact that aggresome formation requires the microtubule network and the microtubule-associated motor, dynein (Wojcik et al., 1996; Garcia-Mata et al., 1999; Johnston et al., 2002). Disruption of the microtubule network, or dynein motor function, was shown to prevent aggresome formation, and led to the appearance of dispersed foci of misfolded protein aggregates throughout cytoplasm (Johnston et al., 1998; Garcia-Mata et al., 1999). These observations led to a model whereby the dynein motor complex, a minus end-directed motor that moves toward the MTOC, acts to collect and transport misfolded and aggregated proteins from the cytoplasm to the aggresome for degradation. However, the mechanism whereby polyubiquitinated misfolded protein aggregates are recognized by, and loaded onto, dynein motors for transport to aggresomes is not known.

Most aggresomes, including those induced by CFTR-ΔF508, are enriched in polyubiquitin. However, some aggresomes, such as those formed by the expression of misfolded GFP-250 (Garcia-Mata et al., 1999) and a mutant superoxide dismutase (SOD) (Johnston et al., 2000), do not contain appreciable polyubiquitin. The differential polyubiquitin content in aggresomes is correlated with the fact that misfolded CFTR-ΔF508 is polyubiquitinated, but GFP-250 and SOD are not (Jensen et al., 1995; Garcia-Mata et al., 1999; Johnston et al., 2000). These observations indicate that not all misfolded protein aggregates processed by aggresomes are polyubiquitinated. However, it is not known how the dynein motor is able to recognize both the polyubiquitinated and nonubiquitinated misfolded proteins, or whether it recruits these two different protein cargos via the same or different mechanism.

In this report, we provide evidence of a functional interaction between HDAC6, dynein motors, and polyubiquitinated misfolded proteins that is essential for aggresome formation. We show that HDAC6 is selectively involved in polyubiquitin-enriched aggresome formation in cultured cells, and also highly concentrated in Lewy bodies in brain tissues affected by Parkinson's disease. Mechanistically, we show that HDAC6 has the capacity to bind both the model polyubiquitinated misfolded protein CFTR-ΔF508 and the dynein motor. We propose that HDAC6 facilitates the loading of polyubiquitinated misfolded cargo to dynein motors for transport to the aggresome. Indeed, loss of HDAC6 impairs the recruitment of polyubiquitinated proteins to dynein, the clearance of misfolded protein aggregates from the cytoplasm, and aggresome formation. These deficiencies cause a hypersensitivity to misfolded protein-induced stress resulting in apoptosis. Our findings uncover a surprising role for a deacetylase in aggresome formation, misfolded protein stress response, and neurodegenerative disease.

Results

HDAC6 Is a Component of Aggresomes

We have previously found that HDAC6 associates with ubiquitinated cellular proteins when proteasome activity is inhibited (J.J. Kovacs and T.-P. Yao, submitted). To further examine the significance of this interaction, we determined the subcellular localization of HDAC6 in response to the proteasome inhibitor MG132. As we have reported, in untreated cells, the immunolocalization of HDAC6 displays a punctate cytoplasmic staining with a specific concentration in the perinuclear region and at the leading edge (Figure 1Aa). Strikingly, treatment of cells with the proteasome inhibitor MG132 causes a dramatic relocation of HDAC6 to a single prominent juxtanuclear structure that is easily discernable as a dark inclusion body under phase contrast microscopy (Figures 1Ae and 1Ah). The morphology and localization of this MG132-induced, HDAC6-enriched inclusion body appear to resemble that of an aggresome. Indeed, proteasome inhibitors are known to induce aggresome formation by preventing the degradation of misfolded proteins (Johnston et al., 1998). We therefore further characterized the observed HDAC6-enriched inclusion body for its localization to the microtubule organization center (MTOC) and the presence of known components of aggresomes: the intermediate filament vimentin, proteasomes, and polyubiquitin. As shown by immunostaining in Figure 1A, the HDAC6-containing inclusion body is localized at the microtubule organizing center (MTOC), as revealed by the presence of γ-tubulin (Figure 1Aj), is surrounded by vimentin (Figure 1Af), and is enriched with both 20S proteasome subunits and polyubiquitin (Figures 1Am and 1Ap). Thus, the HDAC6-positive inclusion bodies exhibit the characteristics of an aggresome.

Aggresome formation is proposed to be a cellular response to increasing levels of misfolded proteins in cells (Johnston et al., 1998). To further investigate whether HDAC6 is involved in the misfolded protein-induced stress response, we treated cells with tunicamycin, DTT, and thapsigargin, all potent agents that induce protein misfolding. All of these ER-stress-inducing agents cause aggresome formation and a prominent relocalization of HDAC6 to this structure (Figures 1Bc–1Bf and data not shown). Together, these results demonstrate that HDAC6 translocates to aggresomes in response to agents that increase levels of misfolded proteins.

HDAC6 Selectively Localizes to Ubiquitin-Positive Aggresomes

Aggresomes can be classified as polyubiquitin-positive and polyubiquitin-negative. To investigate whether HDAC6 is involved in a specific type of aggresome, we examined the localization of HDAC6 in relation to CFTR-ΔF508-induced polyubiquitin-enriched aggresomes and GFP-250-induced polyubiquitin-deficient aggresomes. We used a GFP-CFTR-ΔF508 fusion protein to study CFTR-ΔF508 throughout this report (Johnston et al., 1998). As shown in Figures 2Aa–2Ac, almost all CFTR-

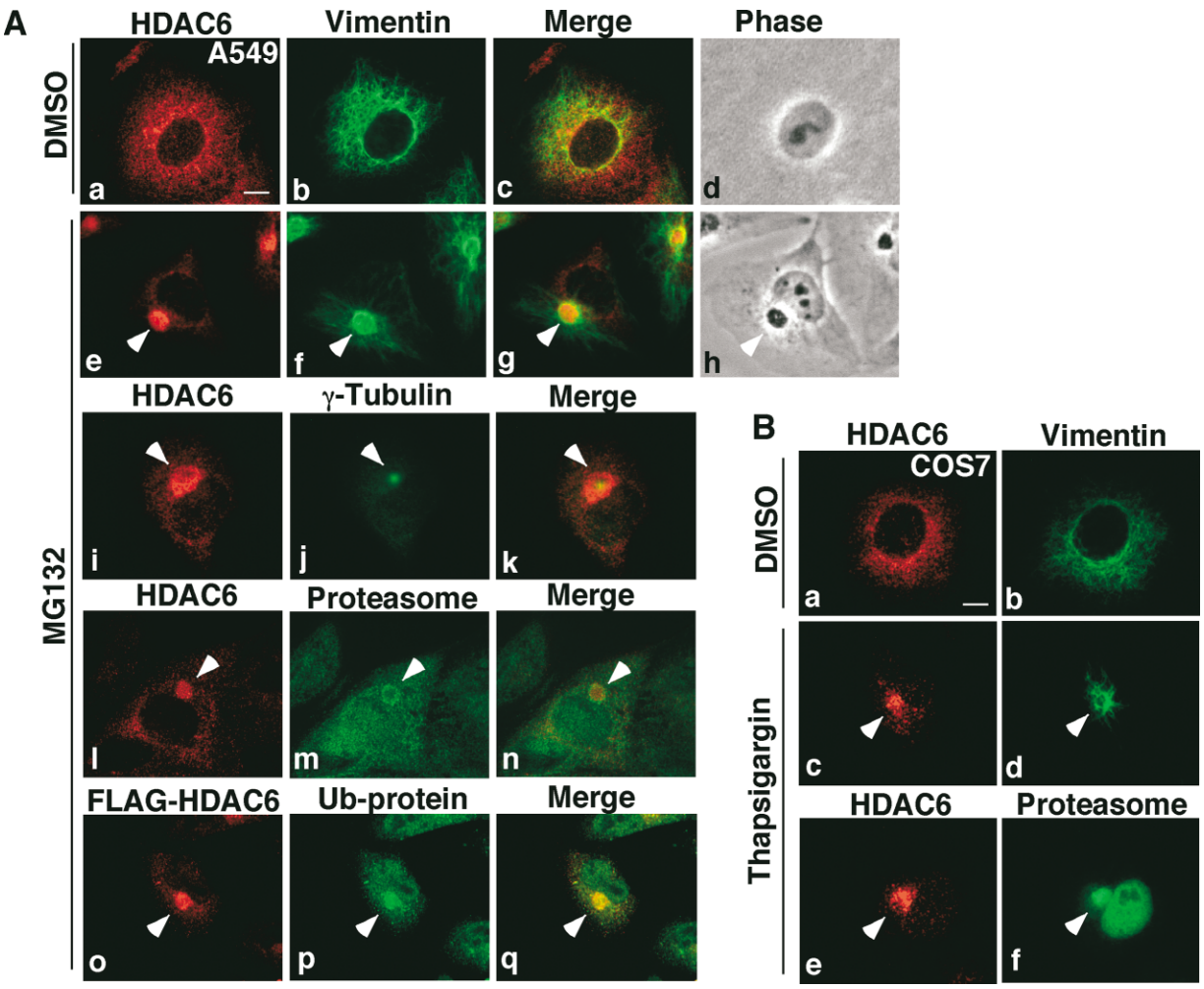

Figure 1. HDAC6 Is a Component of Aggresomes

(A) A549 cells were treated with DMSO or MG132 (5 μM) for 24 hr, and double immunostained with antibodies to HDAC6 (a, e, i, and l; red) and vimentin (b and f; green), γ-tubulin (j; green), or 20S-proteasome (m; green) as indicated. For colocalization with ubiquitin-protein conjugates, A549 cells were transfected with FLAG-tagged HDAC6 expression plasmid and double immunostained with antibodies for FLAG (o; red) and ubiquitin-protein conjugates (p; green). Superimposed confocal images (merge) demonstrate the colocalization of HDAC6 with various aggresome markers (c, g, k, n, and q). Aggresome (arrowheads) formation is also shown by phase contrast microscopy (d and h).

(B) COS7 cells were treated with DMSO or an ER-stress inducing agent, thapsigargin (1 μM, 24 hr), and immunostained with an antibody to HDAC6 (a, c, and e; red), vimentin (b and d; green), or 20S proteasome (f; green). Scale bar = 3 μm.

ΔF508 becomes concentrated in aggresomes when proteasome activity is inhibited. In CFTR-ΔF508-positive aggresomes, an intense signal for HDAC6 is invariably observed by immunostaining (Figures 2Af–2Ah). In fact, prominent colocalization between HDAC6 and CFTR-ΔF508 aggregates can be observed in the cytoplasm even prior to their transport to aggresomes (Figures 2Ab, 2Ag, and 2Al, insets). In stark contrast, little HDAC6 is found in the GFP-250-containing aggresomes, despite the formation of prominent aggresomes upon GFP-250 expression (Figures 2Ae, 2Aj, and 2Ao). The specificity for HDAC6 translocation to CFTR-ΔF508-aggresomes is further supported by the fact that related class II family members HDAC10 and HDAC4 (Guardiola and Yao, 2002; Zhao et al., 2001) are not concentrated in the CFTR-ΔF508 aggresomes (Figures 2Ad, 2Ai, and 2An, and data not shown). These observations demonstrate that HDAC6 is selectively associated with aggresomes that are enriched with a polyubiquitinated misfolded protein.

HDAC6 Binds Polyubiquitinated CFTR-ΔF508

We next addressed how HDAC6 is selectively involved in the polyubiquitin-positive, but not polyubiquitin-deficient, aggresomes. The presence of an ubiquitin binding domain (the BUZ finger) suggests the possibility that the association of HDAC6 with aggresomes might be mediated by its binding to polyubiquitinated misfolded proteins. To test this hypothesis, we asked whether HDAC6 could bind polyubiquitinated misfolded species of CFTR-ΔF508. As shown in Figure 2B, after MG132 treatment, immunoprecipitation of HDAC6 brought down a smear of CFTR-ΔF508-containing high molecular weight proteins (lane 2, top panel). These protein species are apparently ubiquitinated, as they are recognized by an antibody for ubiquitin (lane 2, middle panel).

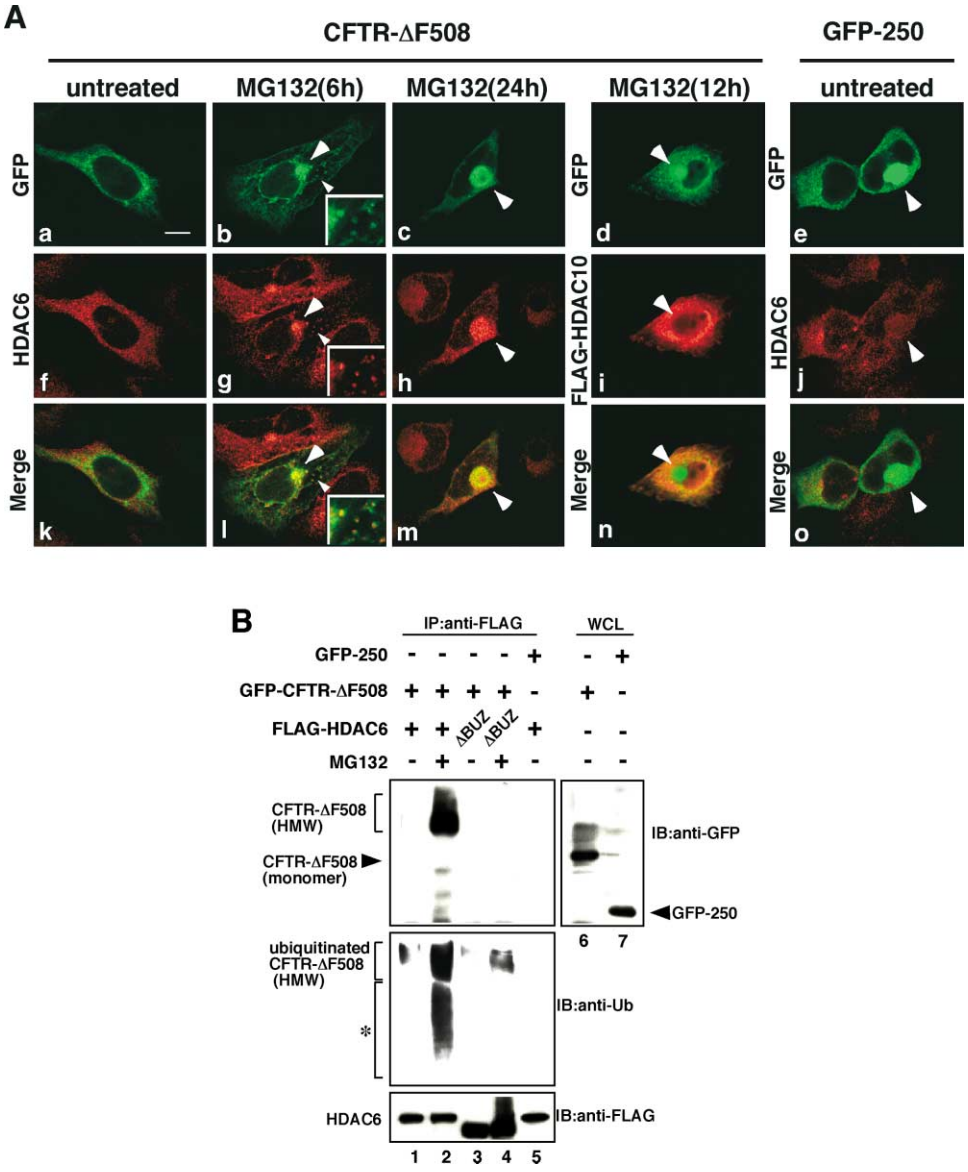

Figure 2. HDAC6 Selectively Localizes to Ubiquitin-Positive Aggresomes through Binding via its BUZ Finger

(A) A549 cells were transfected with expression plasmids for GFP-CFTR-ΔF508 or GFP-250 as indicated. GFP-CFTR-ΔF508 transfected cells were treated with MG132 (5 μM) for 6–24 hr, and visualized with an antibody to GFP (a–d, green) or HDAC6 (f–h; red). Insets in (b), (g), and (l) are high-magnification images of the regions indicated by small arrows that show the colocalization of HDAC6 with small aggregates of CFTR-ΔF508 (l; yellow). Transfected FLAG-HDAC10 was visualized with an antibody to FLAG (i; red). The colocalization of HDAC6 with GFP-250 aggresomes was assessed at 24 hr after GFP-250 transfection (e, j, and o). Large arrowheads indicate aggresomes. Scale bar = 5 μm.

(B) 293T cells were transfected with FLAG-tagged wild-type or ΔBUZ mutant HDAC6, GFP-CFTR-ΔF508, or GFP-250 plasmids, and in the presence or absence of MG132 (5 μM, for 6 hr) as indicated. FLAG-tagged HDAC6 were immunoprecipitated with an antibody to FLAG (M2), followed by immunoblotting with a GFP antibody (top panel), and then reblotted with an ubiquitin antibody (middle panel). The GFP-CFTR-ΔF508 coimmunoprecipitated with wild-type HDAC6 was detected as high molecular weight proteins (HMW) (lane 2, top panel), and appears to be recognized by an antibody to ubiquitin (lane 2, middle panel). Note that other HDAC6-associated ubiquitinated proteins were also detected (lane 2, middle panel, asterisk). The expressions of transfected plasmids were confirmed by immunoblotting (lanes 1–5, bottom panel, and lanes 6–7). WCL: whole cell lysate.

Importantly, HDAC6 does not bind the nonubiquitinated form of CFTR-ΔF508 (lane 6). Further supporting the idea that HDAC6 selectively binds polyubiquitinated CFTR-ΔF508 through the ubiquitin binding BUZ finger, deletion of this motif abrogates this interaction (lane 4, top panel). Wild-type HDAC6 does not interact with GFP-250, which is not subject to polyubiquitination (lane 5). This binding specificity is in complete agreement with the selective presence of HDAC6 in the CFTR-ΔF508 aggresomes but absence from the GFP-250 aggresomes (Figure 2A). Together, these results establish that HDAC6 can specifically bind polyubiquitinated misfolded CFTR-ΔF508 through its C terminus zinc finger, and that this ubiquitin binding capacity might be the basis for the selective involvement of HDAC6 in ubiquitin-enriched aggresomes.

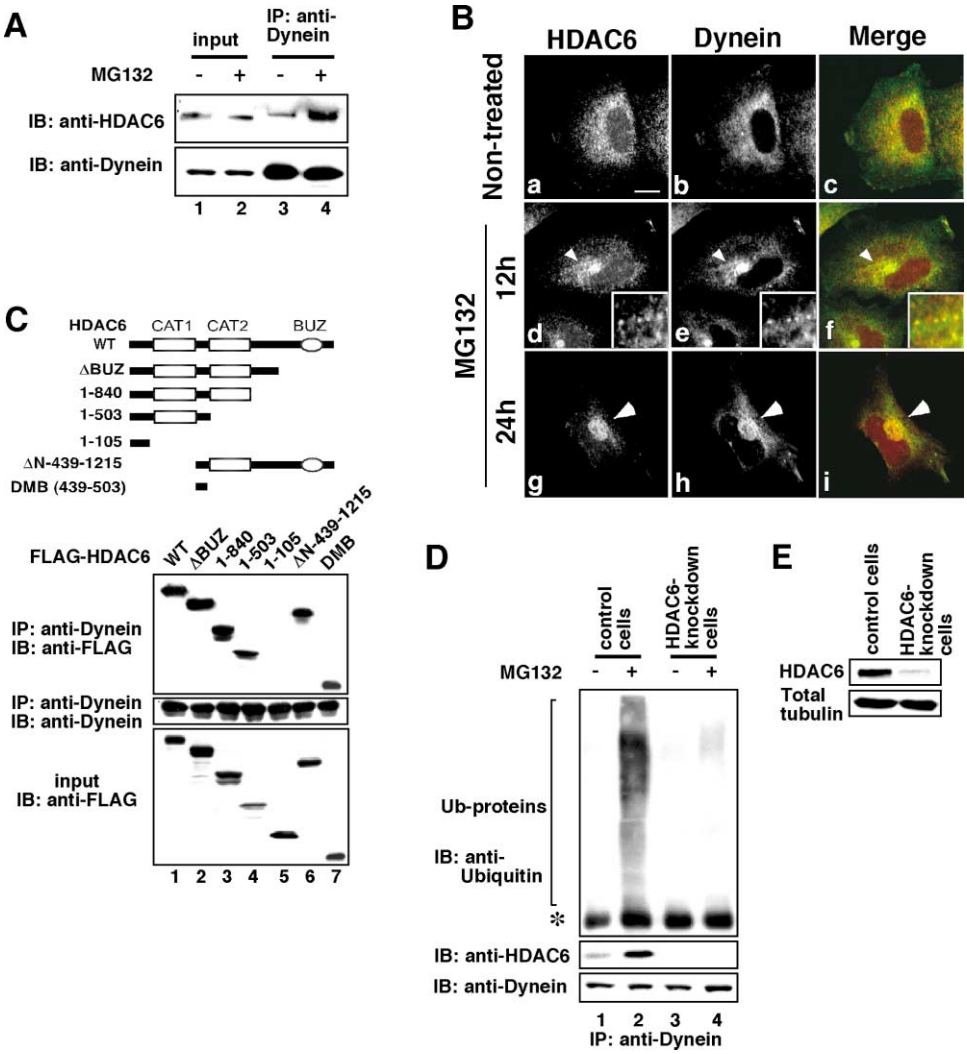

Figure 3. MG132 Treatment Stimulates an HDAC6-Dynein-Ubiquitinated Protein Complex Formation

(A) MG132 treatment induces the interaction of HDAC6 with dynein. A549 cells untreated or treated with MG132 (5 μM, 4 hr) were immunoprecipitated with a dynein antibody (lanes 3 and 4), followed by immunoblotting with an antibody to HDAC6 or dynein as indicated. 10% of total cell lysates used in immunoprecipitation are shown as input (lanes 1 and 2).

(B) A549 cells treated with DMSO or MG132 (5 μM) for 12–24 hr were immunostained with an antibody for HDAC6 (a, d, and g) or dynein (b, e, and h). Superimposed confocal images (merge, yellow) demonstrate the colocalization of HDAC6 and dynein in aggresomes (large arrowheads). Insets in (d)–(f) are high-magnification images of the region indicated by small arrows, and show small foci containing both HDAC6 and dynein (f; yellow). Scale bar = 5 μm.

(C) Identification of dynein motor binding (DMB) motif. FLAG tagged wild-type and HDAC6 deletion mutants were generated as diagramed (top panel). 293T cells transfected with wild-type or mutant HDAC6 constructs were treated MG132 (5 μM, 4 hr). The lysates were immunoprecipitated with an antibody for dynein, followed by immunoblotting with an antibody for FLAG (upper panel), and then reblotted for dynein (middle panel). 10% of total cell lysates used in immunoprecipitation are shown as input (lower panel).

(D) Cell lysates from control or HDAC6 knockdown cells treated or not treated with MG132 (5 μM, 4 hr) were immunoprecipitated with a dynein antibody followed by immunoblotting with antibodies for ubiquitin, HDAC6, and dynein as indicated. Note that dynein immunoprecipitation brought down a series of ubiquitinated proteins from MG132-treated control A549 (lane 2, top panel), but not from HDAC6 knockdown cells (lane 4). Also note an increase in the interaction between HDAC6 and dynein after MG132 treatment (lane 1 and 2, middle panel). Asterisk: immunoglobulin heavy chain.

(E) HDAC6 knockdown cell lines show a significantly lower level of HDAC6. Whole cell lysates from control and HDAC6 A549 knockdown cells were immunoblotted for HDAC6 and total tubulin.

HDAC6 Associates with the Dynein Motor Complex and Recruits Polyubiquitinated Proteins to Dynein

The mechanism by which dynein motors recognize misfolded proteins, including CFTR-ΔF508, is not known. We have previously shown that HDAC6 is associated with microtubules and partially colocalizes with p150^{glued}, a component of the dynein motor complex (Hubbert et al., 2002). As HDAC6 can bind polyubiquitinated misfolded CFTR-ΔF508 (Figure 2B), we investigated the possibility that HDAC6 links the polyubiquitinated misfolded proteins and the dynein motor. To evaluate this hypothesis, we first determined whether HDAC6 physically interacts with the dynein motor complex. Coimmu-

noprecipitation assays demonstrated a constitutive but weak interaction between endogenous HDAC6 and dynein (Figure 3A, lane 3) and p150^{glued} (data not shown). This interaction, however, was markedly stimulated when cells were treated with the proteasome inhibitor MG132 (compare lanes 3 and 4). This observation is consistent with the idea that formation of an HDAC6-dynein complex is stimulated by increasing levels of misfolded proteins in response to MG132 treatment. Supporting this conclusion, immunolocalization studies demonstrated that dynein (Figures 3Bd–3Bf, insets) and p150^{glued} (data not shown) both concentrate in MG132-induced small cytoplasmic aggregate foci and aggresomes and clearly colocalize with HDAC6. By deletion mutant analysis, a 65 amino acid polypeptide adjacent to the second catalytic domain of HDAC6 was found to be sufficient to mediate *d*ynein *m*otor *b*inding (DMB, Figure 3C). Together, these results demonstrate that HDAC6 associates with the dynein motor complex via a specific domain, and this interaction is enhanced by MG132 treatment that induces aggresomes.

To directly test the hypothesis that HDAC6 recruits polyubiquitinated misfolded proteins to the dynein motor, we first determined whether dynein associates with polyubiquitinated proteins. As shown in Figure 3D, immunoprecipitation of endogenous dynein brought down a series of polyubiquitinated proteins from MG132-treated A549 cells, demonstrating that dynein indeed binds polyubiquitinated proteins. To determine if HDAC6 is required for this interaction, we examined the association of dynein and polyubiquitinated proteins in A549 cells stably expressing an HDAC6 siRNA that significantly reduced levels of HDAC6 (Figure 3E). As shown in Figure 3D, the abundance of polyubiquitinated proteins coimmunoprecipitated with dynein was dramatically reduced in the HDAC6 knockdown cells (compare lanes 2 and 4). Collectively, these results are consistent with the idea that HDAC6 mediates the binding of dynein motors to polyubiquitinated misfolded proteins.

HDAC6 Is Required for Aggresome Formation

We have shown that HDAC6 is a component of aggresomes, binds both polyubiquitinated misfolded proteins and the dynein motor, and is required for dynein to recruit polyubiquitinated proteins. These observations led us to hypothesize that that HDAC6 functions to load polyubiquitinated misfolded proteins onto the dynein motor for transport to aggresomes. Were this hypothesis true, HDAC6 should be required for the proper formation of the aggresome.

To test this hypothesis, we analyzed aggresome formation in HDAC6 knockdown cells. We first examined if CFTR-ΔF508-induced aggresomes form normally in HDAC6 knockdown cells. As shown in Figure 4Aa, overexpression of CFTR-ΔF508 readily induces aggresomes of moderate size in control cells 10 hr post MG132 treatment. In contrast, CFTR-ΔF508 appears to be toxic to HDAC6 knockdown cells in the presence of MG132 (detailed discussion in next section). In HDAC6 knockdown cells that survived treatment, no CFTR-ΔF508 aggresome formation was observed (Figure 4Ab). By contrast, GFP-250 aggresomes formed normally in HDAC6 knockdown cells (compare Figures 4Ac and 4Ad). These results demonstrate that HDAC6 is required for aggresome formation induced by ectopic expression of CFTR-ΔF508, but not GFP-250.

To investigate whether HDAC6 is required for aggresome formation induced by endogenous misfolded proteins, we examined aggresome formation in control and HDAC6 knockdown cells in response to MG132 treatment alone. As shown in Figures 4B and 4D, a clearly visible aggresome can be identified in most control A549 cells (~77%) after 24 hr of MG132 treatment. In contrast, under the same treatment, a majority of the HDAC6 knockdown cells (~60%) failed to form a visible perinuclearly localized inclusion body, i.e., aggresome, but instead contained small ubiquitin-positive microaggregates in the cytoplasm (Figure 4Bb). This observation indicates that transport of polyubiquitinated protein aggregates from the peripheral cytoplasm to juxtanuclear aggresomes is compromised in these cells. Importantly, in HDAC6 knockdown cells that do form visible aggresomes in response to MG132 (~40% of cells), aggresome size is significantly smaller than those observed in control cells (compare Figures 4Ca and 4Cc with 4Cb and 4Cd; mean volume shown in 4E). These results support the hypothesis that HDAC6 is required for the transport of misfolded and aggregated proteins necessary for aggresome formation.

To determine the functional domains of HDAC6 required for aggresome formation, we reintroduced wild-type, catalytically inactive, or ubiquitin binding-deficient mutant (ΔBUZ) HDAC6 into the knockdown cells and assessed whether the MG132-induced aggresome formation was restored. To prevent degradation of these expression plasmids by the stable siRNA present in these cells, silent mutations were introduced into the sequences targeted by the HDAC6 siRNA. As shown in Figures 4C–4E, reintroduction of wild-type HDAC6 led to a significant restoration of aggresome formation in HDAC6 knockdown cells (4Ce and 4Cf) as revealed by both an increased percentage of aggresome-containing cells (4D) and the size of aggresomes (4E). This result verifies a pivotal role of HDAC6 in aggresome formation. In contrast to wild-type HDAC6, neither the deacetylase-deficient (DC) nor the ubiquitin binding-deficient HDAC6 mutant (ΔBUZ) had significant activity in restoring aggresome formation (Figures 4D and 4E). Similarly, wild-type, but not mutant, HDAC6 rescues CFTRΔF508 aggresome formation in HDAC6 knockdown cells (data not shown). These results demonstrate that both deacetylase activity and ubiquitin binding activity are required for HDAC6 to regulate aggresome formation.

HDAC6 Is Concentrated in Lewy Bodies of Parkinson's Disease

Lewy bodies share many characteristic features with aggresomes and are hallmarks of several neurodegenerative disorders, including Parkinson's disease and dementia with Lewy bodies (DLB). The requirement of HDAC6 for aggresome formation prompted us to determine whether HDAC6 is also involved in the formation of Lewy bodies. To this end, we performed immunostaining on brain sections from patients with Parkinson's disease or with dementia with Lewy bodies. In these

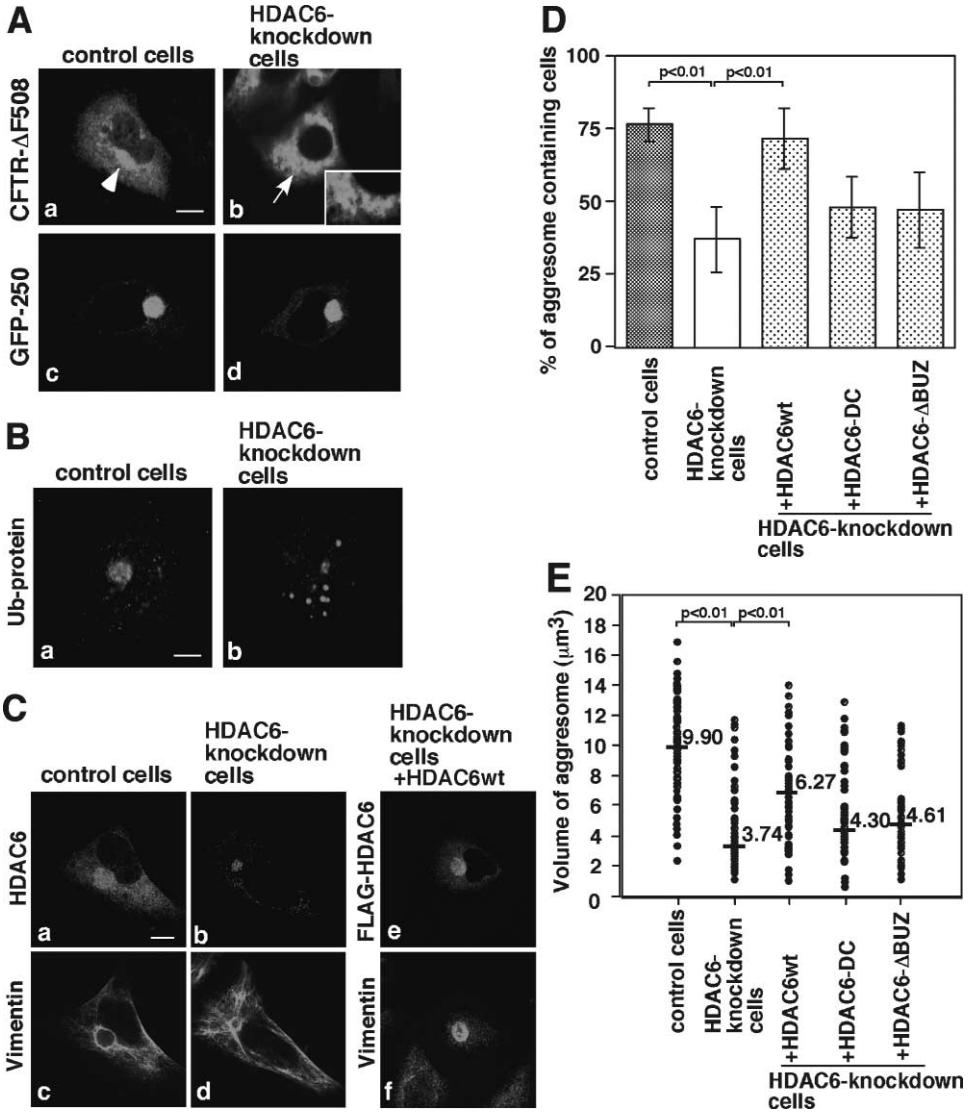

Figure 4. HDAC6 Is Required for Proper Aggresome Formation

(A) CFTR-ΔF508 (a and b), but not GFP-250 (c and d), aggresome formation is impaired in HDAC6 knockdown cells. CFTR-ΔF508 aggresome formation (arrowhead) was assessed in control and HDAC6 knockdown cells as described in Figure 2A, except for a shorter MG132 treatment (10 hr). Inset: higher magnification revealed that CFTR-ΔF508 appeared as microaggregates (arrow) in knockdown cells. Scale bar = 5 μm.

(B and C) MG132-induced aggresome failed to form (B) or formed with reduced size (C) in HDAC6 knockdown cells as revealed by immunostaining with an antibody against polyubiquitin (B), HDAC6, or vimentin (C) as indicated. Note that the presence of polyubiquitin microaggregates in HDAC6 knockdown cells (Bb). Scale bar = 3 μm.

(D and E) Quantification of percentage of cells contain aggresomes (D) and the size of aggresomes (E) in control A549, HDAC6 knockdown cells, and HDAC6 knockdown cells reintroduced with wild-type (wt), catalytically inactive (−DC), or ubiquitin binding deletion mutant (−ΔBUZ) HDAC6 as indicated. Error bars (D) represent standard deviation (SD) calculated from 10 random fields. The mean volume of aggresomes that formed in control cells, knockdown cells, and knockdown cells reintroduced with various HDAC6 constructs is shown in (E). Student's t test was used for statistical analysis.

tissue samples, Lewy bodies are easily identifiable as large inclusion bodies with a high concentration of α-synuclein and ubiquitin. As shown in Figures 5B and 5D, immunostaining with an antibody for HDAC6 reveals intense staining in prominent inclusion bodies present in brain sections from patients with Parkinson's disease and patients with dementia with Lewy bodies. Immunostaining of the adjacent sections demonstrated that these inclusion bodies are Lewy bodies, as they are also positive for α-synuclein and ubiquitin (Figures 5A, 5C, and 5E). These results demonstrate that HDAC6 is also a component of Lewy bodies associated with neurodegenerative diseases.

Loss of HDAC6 Leads to Hypersensitivity to Misfolded Protein-Induced Stress

The failure to process misfolded and aggregated proteins often results in cell death and is a likely a contributor to the pathogenesis of Parkinson's and related neurodegenerative diseases. To determine whether HDAC6

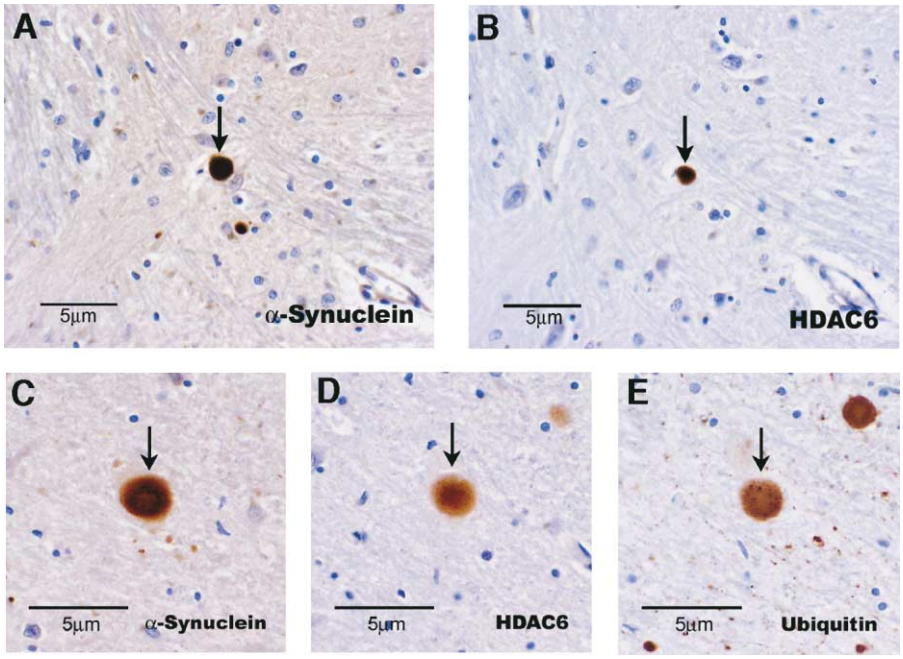

Figure 5. HDAC6 Is Concentrated in the Lewy Bodies of Parkinson's Disease and Dementia with Lewy Bodies

Series of adjacent sections of neocortical Lewy bodies in Parkinson's disease brain (A and B, arrows) and brainstem Lewy bodies in brain sections from patients suffering from dementia with Lewy bodies (C–E, arrows) were immunostained with antibodies for HDAC6, ubiquitin-protein conjugates, and α-synuclein as indicated.

is crucial for cell management of misfolded protein-induced stress, we assessed the viability of HDAC6 knockdown cells in response to accumulation of misfolded proteins induced by inhibition of proteasome activity or by ectopic expression of CFTR-ΔF508. As shown in Figures 6A and 6B, TUNEL assays revealed that MG132 treatment caused a fraction (10% to ~15%) of control A549 cells to undergo apoptosis over time. The same treatment, however, resulted in a significantly higher percentage (20%–30%) of apoptosis in HDAC6 knockdown cells. These results indicate that loss of HDAC6 renders cells more sensitive to misfolded protein stress induced by MG132 treatment.

We next tested whether HDAC6 is specifically required for cellular responses to accumulation of the polyubiquitinated misfolded protein, CFTR-ΔF508. To that end, we transfected GFP-CFTR-ΔF508 or GFP-250 into control and HDAC6 knockdown cells and analyzed their viability. Although GFP-CFTR-ΔF508 expresses and induces aggresome formation in control cells in the presence of MG132 (36 hr treatment), repeated efforts failed to recover viable GFP-CFTR-ΔF508-expressing HDAC6 knockdown cells under the same experimental conditions (data not shown). This result suggests that accumulation of misfolded GFP-CFTR-ΔF508 is extremely toxic to HDAC6 knockdown cells. We subsequently investigated GFP-CFTR-ΔF508 cytotoxicity in the absence of MG132. Under this condition, the expression of GFP-CFTR-ΔF508 in HDAC6 knockdown cells again led to profound cell death (Figures 6Cb, 6Cg, and 6Cl). Quantification shows that more than 75% of GFP-CFTR-ΔF508-transfected HDAC6 knockdown cells underwent apoptosis, compared with only 10% of control cells. In contrast, ectopic expression of GFP-250 in HDAC6 knockdown cells did not lead to appreciable cell death (data not shown), further establishing that the requirement of HDAC6 for viability is specific for polyubiquitinated misfolded CFTR-ΔF508. Importantly, the hypersensitivity of HDAC6 knockdown cells to CFTR-ΔF508 can be significantly alleviated by the reintroduction of wild-type, but not catalytically inactive or ubiquitin binding-deficient, HDAC6 (Figure 6C and 6D). Together, these results demonstrate that HDAC6 is essential for cell viability in response to polyubiquitinated misfolded protein-induced stress.

Discussion

The aggresome has emerged as a key organelle in the clearance of toxic cytoplasmic misfolded protein aggregates often linked with cell death in neurodegenerative diseases. Despite the potential importance of the aggresome in managing stress induced by misfolded and aggregated proteins, and its association with neurodegenerative disease, few protein factors critical for aggresome formation, other than the dynein motor and molecular chaperones, have been identified. Additionally, we do not know how the dynein motor machinery specifically recognizes misfolded and aggregated proteins for transport to aggresomes. In this study, we have identified HDAC6 as a factor crucial for aggresome formation, the recruitment of misfolded protein aggregates to the dynein motor for transport, and cell survival in response to misfolded protein-induced stress.

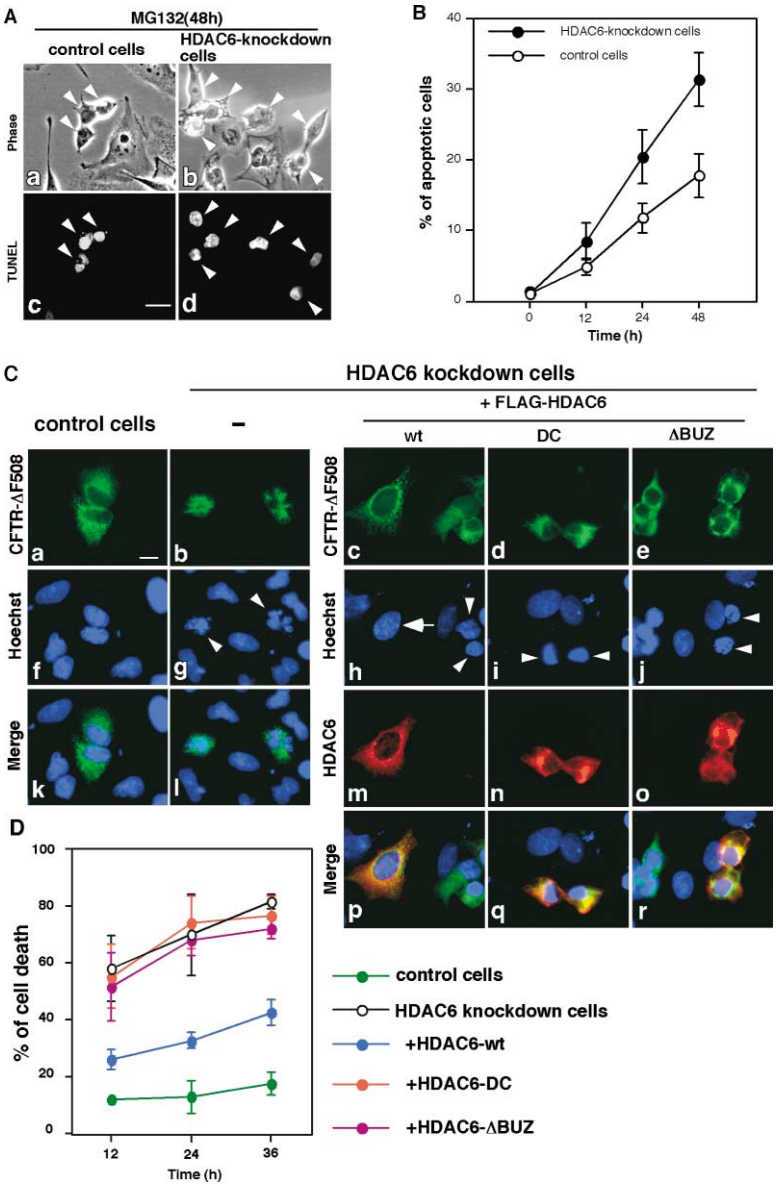

Figure 6. Loss of HDAC6 Leads to Hypersensitivity to Misfolded Protein-Induced Stress

(A) More HDAC6 knockdown cells appear to be refractile as judged by phase contrast microscopy (a and b) and are positive for TUNEL staining (c and d; arrowheads) in response to MG132 treatment. Scale bar = 7.5 μm.

(B) The number of apoptotic cells in control and HDAC6 knockdown cells 0–48 hr after MG132 (5 μM) treatment was counted and graphed. Error bars represent standard deviation (SD) calculated from 3 experiments.

(C) GFP-CFTR-ΔF508 expression plasmids were transfected into A549 control (a, f, and k) or HDAC6 knockdown cells alone (b, g, and l) or with wild-type (c, h, m, and p), catalytic inactive DC (d, i, n, and q), or ΔBUZ mutant HDAC6 (e, j, o, and r). The GFP-CFTR-ΔF508 expressing cells were identified by immunostaining with an antibody for GFP (a–e), and nuclear morphology was visualized by Hoechst 33258 (f–j). Note that many GFP-CFTR-ΔF508-expressing HDAC6 knockdown cells contain condensed and fragmented nuclei (arrowheads in g). The condensed nuclear morphology can be reversed by the reintroduction of wild-type HDAC6 (arrow in h; arrowheads point to condensed nuclei in cells expressing GFP-CFTR-ΔF508 but not HDAC6), but not by DC and ΔBUZ mutant HDAC6 (arrowheads in i and j). The expression of reintroduced FLAG-tagged wild-type and mutant HDAC6 was identified by immunostaining with an antibody for FLAG (m–o, red). Scale bar = 5 μm.

(D) Quantification of CFTR-ΔF508-induced cell death described in (C) by scoring cells with condensed and fragmented nuclei 12–36 hr after transfection. Error bars represent standard deviation (SD) calculated from 3 experiments.

HDAC6 Is a Component in Aggresomes Induced by Misfolded Proteins

Aggresomes eliminate cytoplasmic misfolded protein aggregates by either functioning as proteolysis centers specialized in degrading misfolded and aggregated proteins or as collection centers for their efficient degradation, possibly by an autophagosome-dependent pathway (reviewed in Garcia-Mata et al., 2002). Supporting the central importance of aggresomes as a cellular response to cope with stress triggered by misfolded protein aggregates, aggresomes are induced by diverse agents that cause the accumulation of misfolded proteins (Figure 1). Importantly, treatment with the same agents invariably led to a dramatic translocation of the deacetylase HDAC6 to aggresomes (Figure 1), establishing HDAC6 as a component in aggresomes. The significance of this observation is revealed by the failure of proper aggresome formation in HDAC6-deficient cells (Figure 4), and the greater apoptotic response of these cells to the accumulation of misfolded proteins (Figure 6).

HDAC6: A Bridge between Ubiquitinated-Misfolded Proteins and the Dynein Motor

The capacity to associate with both polyubiquitinated misfolded proteins (Figure 2) and dynein motors (Figure 3) provides the framework to understand how HDAC6 regulates aggresome formation. Dynein motors are proposed to transport misfolded and aggregated proteins from the cytoplasm to the MTOC and are essential for the formation of aggresomes. Indeed, by video time-lapse microscopy, small protein aggregates were found to be actively transported toward aggresomes (Garcia-Mata et al., 1999). However, neither the dynein motor nor its associated dynactin complex has a known ubiquitin binding capacity. It has been a puzzle as to how polyubi-

quitinated misfolded and aggregated proteins are recognized by the dynein motor for their eventual transport to aggresomes. Our study now provides an answer to this puzzle by placing HDAC6 as the bridge between the polyubiquitinated misfolded cargo and the dynein motor. Three lines of evidence support this hypothesis.

First, HDAC6 can bind both polyubiquitinated misfolded proteins and dynein motors. We showed that HDAC6 binds polyubiquitinated misfolded CFTR-ΔF508 via its C terminus ubiquitin binding BUZ domain (Figure 2), whereas it binds the dynein motor through a separate domain (DMB, Figure 3C). Thus, HDAC6 can in theory simultaneously associate with both polyubiquitinated misfolded proteins and the dynein motor, facilitating the loading of cargo to the motor (Figures 2A and 3B). Indeed, we showed that HDAC6 is required for dynein to bind polyubiquitinated proteins (Figure 3D) and for the transport of misfolded proteins to aggresomes (Figures 4A–4C).

Second, HDAC6 colocalizes with both the dynein motor complex and CFTR-ΔF508 in cytoplasmic microaggregates. Immunolocalization demonstrates a clear colocalization between HDAC6 and dynein as well as HDAC6 and CFTR-ΔF508 in small cytoplasmic foci during the course of aggresome formation (Figures 2 and 3). These results indicate that misfolded-CFTR-ΔF508-aggregates, HDAC6, and dynein form complexes prior to transport to the juxtanuclear aggresome. By virtue of its ability to interact with both misfolded protein cargo and the dynein motor complex, HDAC6 can act to collect and transport cytoplasmic misfolded protein aggregates to the aggresome.

Third, misfolded protein aggregates fail to concentrate into aggresomes in HDAC6-deficient cells. If HDAC6 facilitates the loading of misfolded protein aggregates to the dynein motors, one would predict that, in the absence of HDAC6, misfolded proteins will remain as cytoplasmic aggregates, as they cannot be transported to aggresomes. Indeed, instead of a single aggresome, small ubiquitin-containing microaggregates are found throughout the cytoplasm in a majority of HDAC6 knockdown cells treated with MG132 (Figure 4B). Importantly, this is the exact phenotype observed when dynein motor activity is inhibited in the presence of aggresome-promoting agents (Garcia-Mata et al., 1999). These observations place HDAC6 and the dynein motor in a functional complex essential for aggresome formation.

Notably, a catalytically inactive HDAC6 mutant failed to rescue aggresome formation and the hypersensitivity to misfolded proteins in HDAC6 knockdown cells (Figures 4 and 6). This result suggests that HDAC6 does not function simply as an adaptor that physically links polyubiquitinated misfolded proteins to dynein motors, but that a deacetylation event is also required for aggresome formation. As the only known substrate for HDAC6, it is reasonable to speculate that the deacetylation of microtubules by dynein-associated HDAC6 might somehow facilitate the transport of dynein-misfolded protein complexes along microtubules to the MTOC. It is, however, equally possible that deacetylation of substrates other than α-tubulin might be critical for HDAC6 to manage the misfolded proteins. Identifying specific targets of HDAC6 deacetylase activity will be essential to fully understand how reversible acetylation regulates aggresome formation.

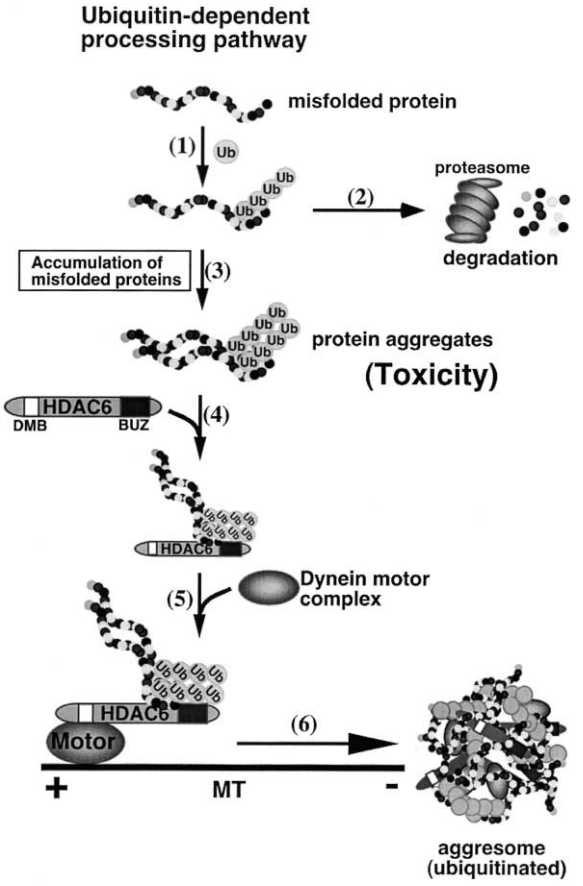

Figure 7. Model for HDAC6-Dependent Processing of Misfolded Proteins and Aggresome Formation

Numbers indicate various steps during aggresome biogenesis as described in the discussion.

HDAC6 and Polyubiquitinated Misfolded Proteins

The involvement of HDAC6 in aggresomes appears to be highly selective. Although HDAC6 is concentrated in aggresomes induced by CFTR-ΔF508, it is not enriched in GFP-250-containing aggresomes. Both types of aggresomes share all the known features associated with aggresomes, except for one key difference: the CFTR-ΔF508 aggresomes contain abundant polyubiquitin, but GFP-250 aggresomes do not (Garcia-Mata et al., 1999). How does HDAC6 achieve this specificity, and how important is this selectivity?

The answer to the first question may lie, in part, in the fact that HDAC6 contains an ubiquitin binding domain, the BUZ finger. Although it does not bind unmodified CFTR-ΔF508 with appreciable affinity, HDAC6 specifically binds polyubiquitinated misfolded CFTR-ΔF508 via the BUZ finger (Figure 2). The selective presence of HDAC6 in CFTR-ΔF508-induced aggresomes might be explained by its specific binding of polyubiquitinated misfolded CFTR-ΔF508 (Figure 2). Since misfolded GFP-250 is not polyubiquitinated, HDAC6 does not recognize

it (Figure 2B). Consequently, HDAC6 is not present in GFP-250 aggresomes (Figure 2A). Further supporting this idea of selectivity, HDAC6 is specifically required for aggresomes induced by CFTR-ΔF508 but dispensable for those induced by GFP-250 (Figure 4A). The importance of this specificity can be easily appreciated by the failure to concentrate misfolded CFTR-ΔF508 to aggresomes in HDAC6 knockdown cells (Figure 4A), leading to massive apoptosis (Figure 6). In stark contrast, no cytotoxicity is associated with GFP-250 expression in the same HDAC6-deficient cells. Thus, HDAC6 appears to be the specific adaptor enabling dynein motors to recognize and associate with the polyubiquitinated misfolded cargo. This conclusion would also predict the existence of specific adaptor(s) for loading the nonubiquitinated misfolded protein cargo, which are also likely associated with neurodegenerative diseases (Johnston et al., 2000).

HDAC6, Aggresomes, Lewy Bodies, and Misfolded Protein-Induced Cell Death

The appearance of intracellular inclusion bodies containing misfolded proteins has emerged as a common theme in many neurodegenerative diseases, including Parkinson's disease, Huntington's disease, and related neurodegeneration caused by expanded polyglutamine (PolyQ) tract in specific disease genes (Tran and Miller, 1999). It is generally believed that the inability to process misfolded and aggregated proteins causes the formation of these inclusion bodies. Whether inclusion bodies are cytotoxic or cytoprotective in the process of neurodegeneration, however, is still under debate. In this regard, aggresomes are of particular interest as they might serve as a model for studying cytoplasmic inclusion bodies like Lewy bodies. Our observation that HDAC6 is highly enriched in both aggresomes and Lewy bodies further establishes the similarity between these two inclusion bodies. In this study, we demonstrate that HDAC6 deficiency results in defects in aggresome formation accompanied by massive cell death in response to misfolded protein-induced stress (Figure 6). Interestingly, inhibition of aggresome formation by disruption of the microtubule network also increases the cytotoxicity associated with misfolded proteins (Taylor et al., 2003). These observations support the idea that aggresome formation is part of a cytoprotective response. HDAC6 could protect cells by facilitating the removal of harmful misfolded protein aggregates from the cytoplasm and concentrating them into aggresomes for efficient processing and degradation. Thus, the formation of disease-associated cytoplasmic inclusion bodies, such as Lewy bodies, is likely a cytoprotective response of affected neurons to combat misfolded protein-induced stress. The presence of HDAC6 in Lewy bodies suggests that HDAC6 is also an important factor for neuronal survival during the process of neurodegeneration. The delineation of the role for HDAC6 in regulating misfolded protein response could have important implications for the treatment of neurodegenerative disease.

Based on our results, we propose the model depicted in Figure 7, which shows that misfolded proteins become polyubiquitinated (1) and normally degraded by proteasomes (2). However, misfolded proteins can escape degradation due to abnormal or pathological conditions and form toxic aggregates (3). These misfolded and aggregated proteins are recognized and bound by HDAC6 through the presence of polyubiquitin chains (4). This allows for the loading of polyubiquitinated misfolded protein cargo onto the dynein motor complex by HDAC6 (5). The polyubiquitinated cargo-HDAC6-dynein motor complex then travels to the aggresome, where the misfolded and aggregated proteins are processed and degraded (6), clearing the cell of cytotoxic protein aggregates. Our results establish the deacetylase HDAC6 as a critical component in managing misfolded protein-induced stress, further expanding the functional repertoire for HDAC family members and reversible acetylation beyond histone metabolism and gene transcription.

Experimental Procedures

Cell Lines and Transfection

All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine. Transfections were performed with FuGENE6 (Roche) or by the calcium phosphate method. A retrovirus-mediated pSUPER RNAi system (OligoEngine) was used to establish the A549 line expressing an RNAi for HDAC6. Silent mutations (G237C, G240A, and A243C) were introduced into wild-type and mutant HDAC6 that were reintroduced into the knockdown cells.

Antibodies and Plasmids

The anti (α)-HDAC6 antibody has been described previously (Hubbert et al., 2002). The following antibodies were used in this study: α-vimentin (V9, H-84 Santa Cruz), α-γ-tubulin (GTU-88, Sigma), α-20S-proteasome (HC8), α-ubiquitin-protein conjugates (Affiniti Research Products), α-dynein (70.1, Sigma), α-ubiquitin (clone P467), α-FLAG (M2, Sigma), α-GFP (clone 7.1 and 13.1, Roche), α-α-synuclein (BD Transduction Laboratories), and α-myc (9E10, Sigma). pCDNA3-FLAG-tagged wild-type and mutant HDAC6 were generated as described elsewhere (J.J. Kovacs and T.-P. Yao, submitted).

Immunostaining

Immunofluorescence was performed as described previously (Hubbert et al., 2002) except for experiments visualizing the 20S-proteasome and ubiquitin-protein conjugates, in which cells were fixed in methanol at −20°C. For immunostaining of Lewy bodies, all tissue from patients that had confirmed postmortem diagnoses of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) was fixed in 10% formalin, paraffin embedded, and serially sectioned at 8 μm thickness. Adjacent slides were stained for α-HDAC6, α-synuclein, and α-ubiquitin-protein conjugates. Vector ABC kit (Vector laboratories) was used to visualize the stain and counterstained with hematoxylin.

Quantification of Aggresome-Containing Cells and Measurement of Aggresome Size

For quantification, ten fields of each sample were randomly selected. The percentage of cells containing aggresomes was counted. For measurement of the volume of aggresome, 75 cells of each sample were randomly selected. The volume of aggresomes was measured using confocal microscopy software (UltraView, Perkin Elmer). Student's t test was used for statistical comparison.

Cell Death Assay

Apoptotic cells induced by MG132 were detected by the TUNEL method using the In Situ Cell Death Detection Kit (Roche). Three hundred cells were analyzed. For CFTR-ΔF508-induced cell death, cell nuclei were visualized by staining with Hoechst 33258 (Sigma). One hundred CFTR-ΔF508-expressing cells were analyzed for the presence of condensed or fragmented nuclei.

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