14-3-3 protein targets misfolded chaperone-associated proteins to aggresomes

Zhe Xu, Kourtney Graham, Molly Foote, Fengshan Liang, Raed Rizkallah, Myra Hurt, Yanchang Wang, Yuying Wu and Yi Zhou*

Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, FL 32306, USA

*Author for correspondence (yzhou@fsu.edu)

Accepted 19 June 2013
Journal of Cell Science 126, 4173–4186
© 2013. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.126102

Summary

The aggresome is a key cytoplasmic organelle for sequestration and clearance of toxic protein aggregates. Although loading misfolded proteins cargos to dynein motors has been recognized as an important step in the aggresome formation process, the molecular machinery that mediates the association of cargos with the dynein motor is poorly understood. Here, we report a new aggresome-targeting pathway that involves isoforms of 14-3-3, a family of conserved regulatory proteins. 14-3-3 interacts with both the dynein-intermediate chain (DIC) and an Hsp70 co-chaperone Bcl-2-associated athanogene 3 (BAG3), thereby recruiting chaperone-associated protein cargos to dynein motors for their transport to aggresomes. This molecular cascade entails functional dimerization of 14-3-3, which we show to be crucial for the formation of aggresomes in both yeast and mammalian cells. These results suggest that 14-3-3 functions as a molecular adaptor to promote aggresomal targeting of misfolded protein aggregates and may link such complexes to inclusion bodies observed in various neurodegenerative diseases.

Key words: Aggresome, 14-3-3, Dynein, BAG3, Adaptor

Introduction

Misfolded proteins are prone to forming aggregates that perturb normal cellular functions and lead to cytotoxicity (Ross and Poirier, 2005). In addition to chaperone-assisted refolding and ubiquitin-proteosome mediated degradation, the aggresome-autophagy dependent clearance has recently been identified as an important cellular response to toxic misfolded proteins (Tyedmers et al., 2010). When proteasome-dependent degradation fails, misfolded proteins can be sequestered into large juxtanuclear inclusion bodies known as aggresomes, which not only help alleviate the toxicity of small aggregates, but also facilitate their clearance by macroautophagy (Garcia-Mata et al., 2002; Kopito, 2000). Aggresome formation is an active process in which misfolded proteins are loaded onto the dynein–dynactin motor complex by protein adaptors for transport along microtubules to the microtubule organizing center (MTOC) (Chin et al., 2010; Ravikumar et al., 2005). While the underlying molecular details are not fully understood, previous studies have identified the dynein motor and molecular adaptors [e.g. histone deacetylase 6 (HDAC6)] as critical components in the aggresome formation process (Kawaguchi et al., 2003; Ravikumar et al., 2005). More recently, heat-shock protein 70 (Hsp70) and its co-chaperone Bcl-2-associated athanogene 3 (BAG3) were shown to be critical for targeting misfolded proteins to aggresomes (Gamerdinger et al., 2011; Zhang and Qian, 2011).

We report here that aggresome formation is profoundly regulated by 14-3-3, a family of ubiquitous proteins that are abundantly expressed in the brain (Bostoen et al., 1982; Moore, 1967). 14-3-3 proteins are highly conserved from yeast to human and consist of seven mammalian isoforms (β, ε, η, γ, τ/θ, ζ and σ) (Rosenquist et al., 2000; Wang and Shakes, 1996). The crystal structures of human 14-3-3 proteins reveal that they exist as homo- and heterodimers, with each monomer comprising nine α-helices organized in an anti-parallel array (Liu et al., 1995; Xiao et al., 1995). Co-crystallization of several ligand-bound 14-3-3 complexes further demonstrate that the 14-3-3 dimer is arranged in such a way that the ligand binding groove runs in opposite directions for each monomer, thereby allowing simultaneous binding of two ligands for one 14-3-3 dimer (Rittinger et al., 1999).

14-3-3 proteins are known to interact with over 200 proteins that contain specific phosphoserine/phosphothreonine motifs (Furukawa et al., 1993; Muslin et al., 1996). Through binding to their target proteins, 14-3-3 participates in a wide variety of biological processes, ranging from transcription to neuronal development (Datta et al., 2000; Fantl et al., 1994; Fu et al., 2000; Peng et al., 1997; Skoulakis and Davis, 1998; Tzivion and Avruch, 2002; Zha et al., 1996). In addition, 14-3-3 proteins have been implicated in a number of neurodegenerative disorders, largely based on the observation that 14-3-3 proteins co-localize with the pathological inclusion bodies associated with these diseases, including Lewy bodies in Parkinson’s disease, neurofibrillary tangles in Alzheimer’s disease, mutant huntingtin aggregates in Huntington’s disease, etc. (Chen et al., 2003; Kawamoto et al., 2002; Umahara et al., 2004). However, it is not known how and why 14-3-3 assembles in these inclusion bodies (Foote and Zhou, 2012).

It has been hypothesized that the presence of 14-3-3 proteins in various inclusion bodies may be a consequence of their associations with disease-related proteins such as α-synuclein (α-Syn), parkin and tau, which are major components of inclusions in certain neurodegenerative diseases (Hashiguchi...
et al., 2000; Ostrerova et al., 1999; Sato et al., 2006). On the other hand, 14-3-3 has been proposed to actively promote the formation of aggresome-like inclusions, thus acting as a sweeper to facilitate the sequestration and deposition of disease-associated toxic proteins (Kaneko and Hachiya, 2006). In support of this hypothesis, previous studies have shown that 14-3-3 plays an important role in aggresome formation. For example, 14-3-3ζ is required for aggresome formation induced by the expression of a polyglutamine-expanded huntingtin protein (Htt103QΔ) in mammalian cells (Omi et al., 2008). Likewise, deletion of Bmh1, which encodes one of two yeast 14-3-3 homologs Bmh1, inhibits aggresomal targeting of another disease-related huntingtin protein (Htt103QP) ectopically expressed in yeast cells (Wang et al., 2009). Current studies, however, have not yet addressed whether the effect of 14-3-3 on aggresome formation is specific for the huntingtin proteins, and more importantly, which molecular step 14-3-3 might participate in the aggresome formation pathway.

In this study, we have determined that 14-3-3 functions as a molecular adaptor to couple chaperone-associated misfolded proteins with dynein motors through its dimeric binding to both BAG3 and the dynein intermediate chain (DIC). 14-3-3 thus plays a critical role in recruiting misfolded protein cargos onto the cytoplasmic motor complex, thereby promoting aggresome formation in response to accumulation of various misfolded proteins. Our findings set a stage for further determination of the molecular detail and functional significance of the 14-3-3-mediated aggresomal targeting complex and provide a foundation to investigate the protective role of 14-3-3 in neurodegenerative diseases.

**Results**

**14-3-3 facilitates the formation of α-Syn aggresomes**

14-3-3 proteins have previously been reported to interact with α-Syn, one of the major components of Lewy bodies (Ostrerova et al., 1999; Sato et al., 2006). To examine the potential role of 14-3-3 in α-Syn aggregation, we adopted an established method to induce α-Syn aggregation by inhibition of proteasome functions in cells expressing α-Syn-EGFP fusion proteins (McLean et al., 2001). Consistent with the previous report, α-Syn-EGFP fusion protein underwent C-terminal cleavage, and treatment of cells with the proteasome inhibitor ALLN resulted in a marked accumulation of C-terminal truncated fragments in the Triton-X-100-insoluble fraction (pellet) (Fig. 1A). Strikingly, the ALLN-induced α-Syn-EGFP fragments were markedly reduced in the pellet from cells co-transfected with pSCM138, which encodes the YFP fused dlopein (dimeric fourteen-three-three peptide inhibitor) that antagonizes the interaction of 14-3-3 proteins with their endogenous partners (Masters and Fu, 2001) (Fig. 1A, bottom panel). Conversely, co-expression of exogenous 14-3-3 led to a moderate increase in the accumulation of insoluble α-Syn-EGFP fragments after ALLN treatment (Fig. 1A, bottom panel). Since the cleavage of α-Syn-EGFP proteins was only moderately affected by 14-3-3 (Fig. 1A, top panel), a major effect of 14-3-3 appears to be enhancing α-Syn aggregation.

Next, we generated a construct encoding a C-terminal truncated α-Syn-EGFP (Δ155), which was previously shown to be a component of the α-Syn-insoluble species and able to form inclusion bodies (McLean et al., 2001). The effect of 14-3-3 on promoting ALLN-induced aggregation of α-Syn-EGFPΔ155 proteins was first confirmed by western blotting (supplementary material Fig. S1). To further characterize the function of 14-3-3 in α-Syn inclusion body formation, we co-transfected α-Syn-EGFPΔ155 with either exogenous 14-3-3 or pSCM138 in cells and examined inclusion body formation microscopically. In line with our biochemical data, a majority of the cells with overexpressed 14-3-3 bore large α-Syn inclusion bodies after a treatment of the proteasome inhibitor (ALLN), while few ALLN-induced inclusion bodies were observed in cells co-transfected with pSCM138 (Fig. 1C). Further quantification showed that overexpressing 14-3-3 in cells gave rise to significantly more frequent inclusion body formation than that in control cells. In contrast, co-transfection of pSCM138 led to a drastic decrease in the percentage of cells containing large inclusion bodies (Fig. 1B).

It is known that overexpression of α-Syn leads to formation of a specific type of inclusions termed aggresome, which is uniquely located in the vicinity of the centrosome and assembled by retrograde transport of dynein motors along the microtubule network (Opazo et al., 2008; Tanaka et al., 2004). Consistently, these perinuclear structures were enriched with vimentin, a known aggresome marker that displayed homogeneous distribution in cells without aggresome formation (Fig. 1C). Thus, the α-Syn-enriched inclusion bodies facilitated by 14-3-3 were aggresomes.

**14-3-3 plays an important role in aggresome formation**

To test whether 14-3-3 is able to promote aggresome formation of other aggregation-prone proteins, we utilized two other well-established aggresome formation models: GFP-250, which spontaneously forms aggresomes when expressed in cells; and a deletion mutant of cystic fibrosis transmembrane conductance regulator (CFTR), CFTRΔF508, which concentrates in aggresomes when proteasome activity is inhibited (García-Mata et al., 1999; Johnston et al., 1998). As shown in Fig. 2A, 14-3-3 overexpression increased the percentage of cells bearing large perinuclear GFP-250 aggresomes. In cells co-transfected with 14-3-3-binding antagonist pSCM138, however, aggresome formation was suppressed and GFP-250 appeared to exist in small aggregates in the cytoplasm. Similarly, GFP-CFTRΔF508 aggresome formation was enhanced in 14-3-3-overexpressed cells, but significantly suppressed in cells co-transfected with pSCM138 (Fig. 2B). These results show that 14-3-3 also promotes aggresome formation of other aggregation-prone proteins, suggesting a general role of 14-3-3 in the aggresome formation process.

**14-3-3 exhibits isoform specificity in promoting the formation of aggresomes**

There are seven homologous 14-3-3 isoforms in mammals. To determine whether 14-3-3 has isoform specificity in promoting aggresome formation, we co-transfected GFP-CFTRΔF508 with each of the 14-3-3 isoforms in cells, and assessed their effects on aggresome formation by analyzing the percentage of cells containing aggresomes after proteasome inhibition. As shown in Fig. 3A, overexpression of 14-3-3γ, η, ε and ζ isoforms did not lead to a statistically significant increase in the formation of GFP-CFTRΔF508-containing aggresome, but overexpression of 14-3-3γ, β and σ had minimal effects. Thus aggresome formation appears to be specifically facilitated by certain isoforms of 14-3-3.
Given that 14-3-3 overexpression resulted in the largest quantitative increase in aggresome formation (Fig. 3A), we further tested its ability in this process by examining aggresome formation in 14-3-3γ-knockdown cells, in which endogenous 14-3-3γ protein level was significantly reduced by transfection of siRNA targeting the 14-3-3γ gene (14-3-3 siRNA) (Fig. 3B). On average, 32% of the 14-3-3γ-knockdown cells transfected with GFP-CFTRΔF508 developed visible perinuclear aggresomes in response to proteasome inhibitor treatment, which is significantly less than that in control cells (55%) under the same treatment (Fig. 3C). Similarly, knockdown of 14-3-3β with 14-3-3 siRNA led to a significant decrease in the formation of GFP-CFTRΔF508-containing aggresomes (Fig. 3B,C). Together, these results confirm the effectiveness of specific isoforms of 14-3-3 proteins in promoting aggresome formation. Thus, we chose to use the γ isoform as exogenously overexpressed 14-3-3 in the subsequent experiments.

14-3-3 promotes the formation of aggresomes in an HDAC6-independent pathway

HDAC6 is a known protein adaptor that selectively promotes aggresome formation of ubiquitylated cargos such as CFTRΔF508, but has little effect on GFP-250-induced polyubiquitin-deficient aggresomes (Kawaguchi et al., 2003). Our data showed that 14-3-3 was positively involved in the aggresome formation of both GFP-250 and GFP-CFTRΔF508, raising a possibility that 14-3-3 functions in an HDAC6-independent pathway. To test this hypothesis, we first asked whether 14-3-3 physically interacts with HDAC6. As assessed by the co-immunoprecipitation assay, there was no apparent interaction between exogenous 14-3-3 and HDAC6 in co-transfected cells (Fig. 4A, lanes 2, 3). In addition, we evaluated the effects of 14-3-3 on CFTRΔF508 aggresome formation in HDAC6-knockdown cells. Similar to the previous report (Kawaguchi et al., 2003), aggresome formation efficiency was
significantly reduced in HDAC6-knockdown cells at basal condition (Fig. 4B). However, overexpression of 14-3-3 was capable of promoting aggresome formation in HDAC6-knockdown cells, and the effect of exogenous 14-3-3 was more pronounced in HDAC6-knockdown cells than that in the control cells (Fig. 4B). On the other hand, co-transfection of the 14-3-3 binding antagonist pSCM138 led to a further reduction of aggresome formation in HDAC6-knockdown cells compared with control cells (Fig. 4B). Taken together, these results indicate that 14-3-3 promotes aggresome formation via a mechanism that is independent of HDAC6.

14-3-3 is a component of aggresomes

To determine the involvement of 14-3-3 in the aggresome formation process, we examined the subcellular localization of 14-3-3 in aggresome-bearing cells by immunocytochemistry. In these experiments, we co-transfected 14-3-3γ with α-SynEGFPΔ155, GFP-CFTRΔF508 or GFP-250. For α-SynEGFPΔ155 and GFP-CFTRΔF508 co-transfected cells, proteasome inhibitor was applied to induce aggresomes. As shown in Fig. 4C, 14-3-3γ was enriched in the aggresomes induced by all three aggregation-prone proteins. These results are consistent with previous findings that 14-3-3 proteins are components of pathological inclusion bodies and indicate that 14-3-3 is transported to aggresomal compartment together with misfolded proteins.

14-3-3 binds to the dynein intermediate chain

Dynein motors serve to transport associated cargos to the aggresomal compartment at the MTOC region. Using the Golgi morphology test (Palmer et al., 2009; Yadav and Linstedt, 2011), we verified that cytoplasmic dynein functions were not perturbed by the inhibition of 14-3-3 in cells (supplementary material Fig. S2). Given that 14-3-3 may function in parallel with HDAC6 to facilitate aggresome formation and it is a component of aggresomes, we hypothesized that 14-3-3 might act as a molecular adaptor to bridge the interaction of misfolded protein cargos and dynein motors. To test this, we first asked whether 14-3-3 associates with dynein. Results of the co-immunoprecipitation assay demonstrated that 14-3-3 interacted with both endogenous and exogenous DIC (Fig. 5A,B). To further determine the molecular details of this interaction, we generated a series of C-terminal truncated DIC constructs and tested their interactions with 14-3-3 by GST pull-down assay. As shown in Figs 5C,D, 14-3-3 binding to DIC was markedly reduced after a removal of 383 amino acids from its C-terminal end, and further deletion of 59 amino acids resulted in a loss of 14-3-3 binding. Similar results were obtained using the co-immunoprecipitation analyses of
tsA 201 cell lysates co-expressing 14-3-3 and these truncated GFP-mDIC constructs (data not shown). Thus, the C-terminal region of DIC appears to be important for its interaction with 14-3-3.

Dimerization of 14-3-3 is a functional requirement in the formation of aggresomes

14-3-3 proteins exist as homo- or heterodimers in cells (Aitken, 2002). In some cellular pathways, 14-3-3 functions as a protein adaptor to bridge the interaction of two different proteins through its dimeric binding (Shen et al., 2003; Tzivion et al., 1998). To investigate whether 14-3-3 dimeric binding is required for promoting aggresome formation, we utilized several 14-3-3 dimerization-deficient mutants, including MM14-3-3, which bears site-directed mutations in both dimerization interfaces, and WM14-3-3 or MW14-3-3, which has mutations in one of the sites (Zhou et al., 2003). We have previously shown that all three mutants fail to form 14-3-3 dimers, but retain the ability to bind substrates (Zhou et al., 2003). When co-transfected with CFTRΔF508 into cells, none of the dimerization-deficient 14-3-3 mutants exhibited statistically significant effect on promoting aggresome formation (Fig. 6A), suggesting that 14-3-3 dimerization is a functional requirement for its role in aggresome formation.

Furthermore, we assessed the role of 14-3-3 dimerization in aggresome formation in yeast cells. Bmh1, one of the two 14-3-3 homologs in yeast, is known to be essential for aggresome formation by the expression of Huntington’s disease protein Htt103QP in yeast cells (Wang et al., 2009). Consistent with the report, we found that expression of Htt103QP-GFP led to formation of aggresomes and was not toxic in wild-type budding yeast cells (Fig. 6B,Ca). As the levels of WT and MM14-3-3 proteins in bmh1Δ mutant cells were comparable (Fig. 6D, bottom panel), the difference in their rescuing abilities was not likely due to the instability of the MM14-3-3 protein, which has been observed in the fruit flies (Messaritou et al., 2010). In addition, we assessed the extent of aggresome formation by western blot analysis of the relative distribution of Htt103QP proteins in the pellet and supernatant fractions of the yeast cell extracts (Fig. 6D). Co-transformation of the WT14-3-3, but not the dimerization-deficient 14-3-3 mutant (MM14-3-3), in bmh1Δ cells shifted more Htt103QP proteins into the pellet fraction (Fig. 6D, top panel), which corresponds to the successful rescue of Htt103QP aggresome formation by wild-type 14-3-3. Taken together, these data demonstrate that dimerization of 14-3-3 is required to promote aggresome formation in both yeast and human cells.

14-3-3 interacts with the Hsp70 co-chaperone BAG3 in a phosphorylation-dependent manner

Subsequently, we addressed how 14-3-3 might associate with cargo proteins. Given that 14-3-3 promoted aggresome formation of several different aggregation-prone proteins, it is possible that the potential 14-3-3-cargo interaction is mediated by certain intermediate proteins, such as chaperones which are usually associated with misfolded proteins (Zhang and Qian, 2011). A previous proteomic analysis has shown that 14-3-3 interacts with Hsp70 and its co-chaperone BAG3 (Ge et al., 2010a). Since
both proteins are known to play important roles in aggresome formation (Gamerdinger et al., 2011; Zhang and Qian, 2011), we carried out studies to further characterize the binding of 14-3-3 to Hsp70 and BAG3. Our co-immunoprecipitation results demonstrated that 14-3-3 interacted with exogenous BAG3 and Hsp70 in co-transfected cells (Fig. 7A, lane 2; Fig. 7B, lane 2). However, exogenously expressed BAG3 significantly increased the amount of Hsp70 co-immunoprecipitated with 14-3-3 (Fig. 7B, lanes 1, 2), while overexpressing Hsp70 had little effect on the interaction between 14 and 3-3 and BAG3 (Fig. 7A, lanes 1, 2). Thus, the interaction between 14 and 3-3 and Hsp70 is likely bridged by BAG3.

BAG3 contains two putative 14-3-3 binding motifs (RSQS\textsuperscript{136} and RSQS\textsuperscript{173}) located upstream from its BAG domain (for Hsp70 binding). To define the molecular details of the interaction between 14 and 3-3 and BAG3, we assessed the participation of these putative motifs in 14-3-3 binding by site-directed mutagenesis. As shown in Fig. 7C, mutation of the key serine residue in the first motif (S136A) of BAG3 significantly increased the amount of Hsp70 co-immunoprecipitated with 14-3-3 (Fig. 7B, lanes 1, 2), while overexpressing Hsp70 had little effect on the interaction between 14 and 3-3 and BAG3 (Fig. 7A, lanes 1, 2). Thus, the interaction between 14 and 3-3 and Hsp70 is likely bridged by BAG3.

To determine whether phosphorylation of BAG3 is important for its binding to 14-3-3, we treated the cell lysates of 14-3-3 and BAG3 co-transfected cells with alkaline phosphatase, and assessed its effect on the 14-3-3–BAG3 interaction by co-immunoprecipitation and western blot analyses. As shown in Fig. 7D, 14-3-3 binding to BAG3 was decreased by the phosphatase treatment. Moreover, we incubated recombinant BAG3 proteins with tsA201 cell extract in the presence of ATP and Mg\textsuperscript{2+}, and subsequently mixed them with recombinant 14-3-3. Robust 14-3-3 binding was observed when BAG3 protein was subjected to the in vitro phosphorylation reaction by cell extract (Fig. 7D, right panel). Collectively, these results demonstrate that BAG3 phosphorylation is critical for 14-3-3 binding.

14-3-3 mediates the association of BAG3 with dynein

BAG3 has been reported to promote aggresome formation by coupling Hsp70-bound substrates to the dynein complex (Gamerdinger et al., 2011). Given that 14-3-3 interacts with both BAG3 and dynein, we investigated whether 14-3-3 plays a role in the association of BAG3 with dynein. For consistency, these experiments were conducted using approaches as described by Gamerdinger et al. As shown in Fig. 8A, exogenously expressed BAG3 was pulled down by co-transfected GST-DIC. Interestingly, overexpression of 14-3-3 increased the amount of BAG3 co-precipitated with GST-mDIC (Fig. 8A, lanes 1, 2), BAG3 being reported to promote aggresome formation by coupling Hsp70-bound substrates to the dynein complex (Gamerdinger et al., 2011). Given that 14-3-3 interacts with both BAG3 and dynein, we investigated whether 14-3-3 plays a role in the association of BAG3 with dynein. For consistency, these experiments were conducted using approaches as described by Gamerdinger et al. As shown in Fig. 8A, exogenously expressed BAG3 was pulled down by co-transfected GST-DIC. Interestingly, overexpression of 14-3-3 increased the amount of BAG3 co-precipitated with GST-mDIC (Fig. 8A, lanes 1, 2),
Fig. 5. 14-3-3 interacts with dynein-intermediate chain. (A,B) 14-3-3 co-immunoprecipitates with both endogenous dynein-intermediate chain (DIC) (A) and exogenously transfected GFP-mDIC or GST-mDIC (B) in tsA201 cells. Exogenously expressed 14-3-3-HA was immunoprecipitated with an anti-HA antibody. (C) Deletion analyses reveal the region in DIC that is crucial for 14-3-3 binding. GFP-tagged full-length or C-terminal-truncated mDIC-deletion mutants were expressed in tsA201 cells. Their interactions with 14-3-3 were assessed in GST pull-down assays using GST-14-3-3, and in western blots (top panel). (D) Diagram that summarizes the effect of progressive truncation of the DIC C-terminus on its binding to 14-3-3.

Fig. 6. Dimerization of 14-3-3 is required for aggresome formation. (A) Quantification of GFP-CFTRAF508 induced aggresome formation in A549 HDAC6-knockdown cells that had been co-transfected with WT14-3-3, dimerization-deficient mutant MM14-3-3, MW14-3-3, WM14-3-3 or a control vector (**P < 0.001, n=5). (B) Expression of WT14-3-3 but not the dimerization-deficient mutant 14-3-3, suppresses the Htt103QP-induced growth defects in bmh1Δ cells. WT and bmh1Δ yeast cells with integrated P GALFLAG-Htt103QP-GFP plasmids were transformed with vector WT14-3-3 or MM14-3-3 plasmids. The saturated transformants were 10-fold diluted and then spotted on either glucose or galactose plates to examine the growth after 3-day incubation at 30 °C. (C) Representative fluorescence images of WT and bmh1Δ yeast cells with P GALFLAG-Htt103QP-GFP plasmids that were incubated in galactose medium. Transformation of WT14-3-3 (c), but not MM14-3-3 (d), restores aggresome formation in bmh1Δ cells. Aggresomes are shown as large green dots. Scale bar: 3 μm. (D) The yeast cells were incubated in galactose medium for 12 h, and separated into Triton X-100-soluble (supernatant) and -insoluble (pellet) fractions. Samples from both fractions were analyzed by western blotting using anti-FLAG (for Htt103QP) or anti-HA (for expressed WT and MM D14-3-3Δ-HA) antibody, respectively. Pgk1 was used as a loading control.
while the interaction between DIC and BAG3 was totally eliminated in the cells co-transfected with the 14-3-3 binding antagonist pSCM138 (Fig. 8A, lane 3). In addition, the DIC-BAG3 association was either eliminated or reduced for the two BAG3 mutants that were deficient in 14-3-3 binding (Fig. 8B), providing further evidence that the interaction between 14 and 3-3 and BAG3 is critical for the BAG3 and dynein association.

To reveal the hierarchy of the 14-3-3–BAG3–DIC protein complex, we conducted in vitro binding assays using commercially acquired recombinant BAG3, 14-3-3 and bacterially generated GST-mDIC fusion proteins. 14-3-3 co-precipitated with GST-mDIC (Fig. 8C, lanes 1, 3). However, only background level of recombinant BAG3 proteins was detected in the GST-mDIC beads when BAG3 was not subjected to in vitro phosphorylation (Fig. 8C, lanes 3, 4). Interestingly, GST pull-down of BAG3 was markedly enhanced after recombinant BAG3 was incubated with tsA201 cell extract along with ATP (Fig. 8C, lane 1). No such enhancement was observed in the absence of recombinant 14-3-3 and BAG3 is regulated by phosphorylation. (Left panels) co-immunoprecipitation of 14-3-3 and BAG3 is reduced by alkaline phosphatase (AP) treatment of cell lysates. (Right panels) In vitro phosphorylation of recombinant BAG3 with crude tsA201 cell extract significantly enhances its binding to 14-3-3γ, as assessed by co-immunoprecipitation and western blotting. For controls, either ATP (lane 2) or recombinant 14-3-3γ (lane 3) was omitted.

The interaction between 14 and 3-3 and BAG3 is crucial for the formation of aggresomes

To investigate the role of 14-3-3 and BAG3 interaction in aggresome formation, we used siRNA targeting Bag3 to knockdown endogenous BAG3 protein in cells. Consistent with the previous report, aggresome formation was suppressed in cells treated with BAG3 siRNA (Fig. 9A, middle panels). Interestingly, overexpression of 14-3-3 no longer enhanced aggresome formation in cells transfected with BAG3 siRNA (Fig. 9A, left panels), suggesting that exogenous 14-3-3 requires sufficient BAG3 to further promote aggresome formation. In addition, virtually no aggresome formation was observed (less than 5%) in cells co-transfected with both BAG3 siRNA and the 14-3-3-binding antagonist pSCM138 (Fig. 9A, right panels).

Next, we directly tested whether binding to 14-3-3 is a functional requirement for BAG3 to promote aggresome formation. In these experiments, we reintroduced BAG3-siRNA-resistant wild-type or...
14-3-3-binding-deficient Bag3 cDNAs into the BAG3-knockdown cells and assessed their effects on restoring aggresome formation. As shown in Fig. 9B, reintroduction of wild-type BAG3 led to a significant recovery of aggresome formation in BAG3-knockdown cells which were co-transfected with exogenous 14-3-3, control vector or pSCM138. In contrast, reintroduction of the 14-3-3-binding-deficient BAG3 mutant failed to enhance aggresome formation in BAG3-knockdown cells regardless of their levels of functioning 14-3-3 (Fig. 9C). Thus, these data provide strong evidence that 14-3-3 binding to BAG3 is crucial for aggresomal targeting of misfolded proteins.

**Discussion**

14-3-3 functions in an HDAC6-independent pathway

Loading misfolded protein aggregates onto the dynein–dynactin motor complex by protein adaptors is a key step for aggresome formation in cells (Olzmann et al., 2007). Previous studies have identified HDAC6 as one of the protein adaptors that couples ubiquitylated substrates to the dynein motor (Kawaguchi et al., 2003). Our data, however, suggest that 14-3-3 functions in a HDAC6-independent pathway to promote aggresome formation.

First, we identified the positive role of 14-3-3 in promoting aggresome formation of several different aggregation-prone proteins, including both ubiquitylated and non-ubiquitylated cargos such as CFTRΔF508 and GFP-250 respectively (Gamerdinger et al., 2009; Garcia-Mata et al., 1999; Johnston et al., 1998). While HDAC6 is known to specifically promote aggresomal targeting of ubiquitylated cargos, 14-3-3 appears to have a broader effect than HDAC6 on aggresome formation.

Second, although 14-3-3 has been reported to interact with some members of the HDAC protein family, our co-immunoprecipitation analysis did not reveal any apparent interaction between 14-3-3 and HDAC6 (Fig. 4A). As 14-3-3 generally functions by binding to its target proteins, it is unlikely that 14-3-3 acts in conjunction with HDAC6 to promote aggresome formation.

Third, we found that aggresome formation is not only effectively promoted by exogenously expressed 14-3-3, but also significantly suppressed by co-transfection of 14-3-3 binding antagonist in HDAC6-knockdown cells, indicating that HDAC6 may not be required in 14-3-3-mediated aggresome formation pathway. We also showed that the aggresome formation efficiency in cells without both sufficient HDAC6 and functioning 14-3-3 is significantly lower than that in pSCM138 transfected control cells (Fig. 4B). This likely reflects the aggresome promoting effect of endogenous HDAC6 in the absence of 14-3-3. Taken together, these observations suggest that 14-3-3 and HDAC6 function in separate molecular pathways for aggresome formation.

14-3-3 bridges the association of misfolded proteins with the dynein motor

Our biochemical and functional studies support the hypothesis that 14-3-3 functions as a novel molecular adaptor to promote aggresome formation. Based on these data, we propose a working model for the 14-3-3-mediated aggresome formation pathway. As depicted in Fig. 10, misfolded proteins are usually refolded with the assistance of molecular chaperons and/or degraded by the ubiquitin-proteasome system. Under proteolytic stress, both ubiquitylated and non-ubiquitylated misfolded proteins are associated with 14-3-3 via its binding to BAG3. This allows
the loading of the cargo complex onto the dynein motor through the interaction between 14 and 3-3 and DIC. In this pathway, dimeric binding of 14-3-3 is required for the recruitment of misfolded proteins to the dynein motor. The complex containing misfolded proteins, Hsp70, BAG3, 14-3-3 and dynein motors then transports along microtubule to the aggresome at the MTOC region for processing and degradation by macroautophagy.

This model builds on a recently proposed aggresome-targeting pathway mediated by Hsp70 and BAG3 (Gamerdinger et al., 2011). We have provided several lines of evidence to demonstrate the important role of 14-3-3 proteins in this process. Firstly, we identified the interaction between 14 and 3-3 and DIC, and characterized the molecular complex consisting of 14-3-3, BAG3 and Hsp70. Secondly, we found that the BAG3-dynein association requires the intact 14-3-3 binding motifs in BAG3. We further showed that loading of both BAG3 and misfolded proteins onto the dynein motor is regulated by the level of functioning 14-3-3 proteins in the cell. Thirdly, we determined that exogenous 14-3-3 is ineffective in promoting aggresome formation without sufficient BAG3, while BAG3 mutants deficient in 14-3-3 binding fail to rescue the aggresome formation defect when reintroduced into BAG3-knockdown cells. Thus, 14-3-3 appears to be a key linker between chaperone associated misfolded proteins and the dynein motor.

In addition, we showed that 14-3-3 binding to BAG3 can be regulated by BAG3 phosphorylation. This is consistent with our site-directed mutagenesis studies demonstrating the importance of BAG3 phosphoserine containing motifs in mediating 14-3-3 binding. Based on a previous phosphoproteomic analysis, BAG3 is one of the many proteins that exhibit enhanced phosphorylations upon proteasome inhibition, but the phosphorylation of the two key serine residues (S136 and S173) was not identified in that study (Ge et al., 2010b). In view of the potential role of protein phosphorylation in regulating the formation and clearance of
aggresomes (Watabe and Nakaki, 2011), it is important for future studies to reveal the cellular factors (e.g. protein kinases) that regulate phosphorylation of the putative 14-3-3 binding motifs in BAG3, particularly in response to misfolded protein stress.

On the other hand, we confirmed that 14-3-3 homolog Bmh1 is critical for aggresome formation in yeast cells (Wang et al., 2009). Similar to what we found in mammalian cells, dimerization of 14-3-3 is required to facilitate aggresome formation in yeast cells, suggesting that 14-3-3 may also function as a molecular adaptor in the yeast aggresome formation pathway. However, a BAG3 homolog is not found in yeast cells. Thus, it will be interesting to further define the molecular details of 14-3-3-mediated aggresome formation cascade in yeast cells, and particularly how 14-3-3 associates with misfolded proteins without a BAG3 homolog.

14-3-3 in the protein quality control system

In Drosophila cells, 14-3-3 is upregulated by transcriptional activation in response to heat exposure, while overexpression of 14-3-3 led to the resolubilization of several protein aggregates under heat-induced condition (Yano et al., 2006). These behaviors of 14-3-3 are very similar to that of molecular chaperones such as heat shock proteins. In fact, 14-3-3 proteins have previously been referred as molecular chaperones (Vincenz and Dixit, 1996). In support of this notion, recent biophysical studies have also demonstrated that 14-3-3 prevents protein aggregation following chemical stress (Williams et al., 2011).

We report here that 14-3-3 plays a positive role in aggresomal targeting of various aggregation-prone proteins. This is consistent with previous studies showing that 14-3-3 promotes aggresome-like inclusion body formation when the chaperone and proteasome systems are compromised. Under such conditions, 14-3-3 likely functions as an adaptor protein rather than a molecular chaperone. It acts by binding to both the chaperone proteins (BAG3 and Hsp70) and the dynein motor, thereby promoting aggresome formation. In this molecular cascade, dimeric binding of 14-3-3 is key to bridging chaperone-bound misfolded proteins and the dynein motor. In contrast, 14-3-3 dimerization is not a functional requirement for its chaperone-like activity. In fact, monomeric 14-3-3 is reported to display higher chaperone-like activity than its dimeric counterpart or even a chaperone HspB6 (Sluchanko et al., 2012).

Fig. 10. Model for 14-3-3-mediated aggresome-targeting pathway. Several steps of this process are described in the Discussion.
Aggresome has been recognized as a cytoprotective structure that facilitates the removal of toxic misfolded proteins and aggregates from the cytoplasm by concentrating them for efficient processing and degradation. Hence, 14-3-3 proteins appear to involve in different aspects of protein quality control system to prevent the accumulation of misfolded proteins. They can either facilitate resolubilization of protein aggregates under mild stress or promote aggresome-like inclusion body formation when chaperone and proteasome systems are overwhelmed.

14-3-3 and neurodegenerative diseases
14-3-3 proteins are known to colocalize with inclusion bodies found in a number of neurodegenerative diseases, which range from Parkinson’s disease to Amyotrophic Lateral Sclerosis (Chen et al., 2003; Kawamoto et al., 2004; Kawamoto et al., 2002; Richard et al., 2003; Ubl et al., 2004). We showed here that 14-3-3 is a component of aggresomes induced by various aggregation-prone proteins, because it is one of the key factors in the aggresomal targeting cascade. Moreover, we found that 14-3-3 exhibits isoform specificity in promoting aggresome formation, and the 14-3-3 isoforms (γ, η, ε and ζ) which are effective in aggresomal targeting correspond to the particular isoforms previously identified in pathological inclusions such as Lewy bodies (Kawamoto et al., 2002; Ubl et al., 2002). Considering the similar characteristics between aggresomes and inclusion bodies, our findings provide a plausible explanation for the presence of 14-3-3 in pathological inclusion bodies and suggest a positive role of 14-3-3 in the formation of the aggresome-like structures during neurodegeneration.

It is well documented that 14-3-3 promotes cell survival via different signaling pathways such as suppressing apoptosis and reducing cytotoxicity of neurotoxins (Xing et al., 2000; Yacoubian et al., 2010; Zha et al., 1996). Our studies revealed another cytoprotective mechanism through which 14-3-3 facilitates the targeting of toxic protein aggregates into aggresome-like structures. As 14-3-3 proteins are highly enriched in the brain, they could potentially play important roles in neuroprotection by regulating responses to misfolded proteins commonly associated with neurodegenerative diseases.

Materials and Methods
Cell culture and transfection
CHO, tsA201 and AS49 (wild-type and HDAC6-knockdown) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were performed by using the Calcium Phosphate method, FuGENE 6 (Promega) reagent or X-tremeGENE 9 (Roche) reagent.

Plasmids and antibodies
The CDNAs encoding various isoforms of 14-3-3 were generated from a human brain cDNA library (Clontech) by PCR amplification, and then subcloned into pcDNA3 with a C-terminal hemagglutinin (HA) tag. GST-14-3-3 was constructed as reported previously (Zhou et al., 1999). The EGFP-fused γ-Syn cDNA was constructed by fusing EGFP to the C-terminus of γ-Syn coding region in the pEGFP-N1 vector. The plasmids for pSCM138 and pSCM174 were provided by Haian Fu, GFP-250 by Elizabeth S. Sztul, GFP-HDAC6 by Yi Qu, and GFP-mDIC and GST-mDIC by Zhong Sheng. The deletion mutants of the GFP-mDIC were constructed by introducing a stop codon into the cDNA at appropriate locations using GFP-mDIC as template by Quikchange strategy (Stratagene). A similar strategy was used to construct deletion mutant of γ-Syn-EGFPFA155. The mammalian GST-mDIC expression construct was generated by cloning the full length mouse dynen-intermediate chain 1 (Dynclin) cDNA into a pEFG vector. The His-HSPA1A construct was a gift of Harm Kampina (Addgene plasmid # 19537). The BAG3 construct was generated by cloning a human BAG3 cDNA into the pcMV6-AC vector (Origene). Point mutants of BAG3 were generated by site-directed mutagenesis using Quickchange strategy (Stratagene). The S136A mutation in BAG3 (S136A BAG3) reduced binding to 14-3-3, the S173A mutation (S137A BAG3) abolished binding to 14-3-3 altogether.

The SODI-GFP and SODOI -GFP constructs were gifts of Elizabeth Fisher (Addgene plasmid # 26407 and 26410). All constructs were verified by DNA sequencing analysis.

The following antibodies were used in this study: polyclonal anti-γ-Syn antibody (#2642, Cell Signaling Technology), monoclonal anti-vimentin antibody (#6630, Sigma-Aldrich), monoclonal anti-HA antibody, monoclonal anti-flag M2 antibody (Sigma-Aldrich), monoclonal anti-dynein (intermediate chain) antibody (DS167, Sigma-Aldrich), monoclonal anti-GAPDH antibody (MCA-1D4, Stemcell Technologies), monoclonal anti-GFP antibody (NeuroMab), polyclonal anti-GFP antibody (sc-8334, Santa Cruz), polyclonal anti-BAG3 antibody (Proteintech), polyclonal anti-HSC70/HSP70 antibody (pAb, Enzo Life Sciences), polyclonal anti-14-3-3γ antibody (C-16, Santa Cruz), homemade monoclonal anti-ID4 antibody, polyclonal anti-Gr1-1 antibody (Abcam), polyclonal IRDye 800CW Conjugated Goat anti-mouse IgG (H’L, LI-COR) and polyclonal IRDye 800CW Conjugated Goat anti-rabbit IgG (H’, LI-COR).

Immunoprecipitation
Cells were lysed at 4°C using lysis buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM NaF, 25 mM KCl, 10 mM EDTA, 1 mM NaVO4, 1 mM DTT, protease inhibitor cocktail with 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A) containing 1% Triton X-100. Cell lysates were separated by centrifugation at 14,000 rpm for 10 min into Triton-X-100-soluble and -insoluble fractions. Supernatants were incubated with protein A/G agarose beads at 4°C for 1 h. Following by centrifugation at 4000 rpm for 5 min, supernatants were transferred to new tubes and incubated with proper antibodies for 2 h at 4°C. 50 μl A/G beads were added into each tube and incubated overnight at the same temperature. Immunoprecipitates were washed three times with lysis buffer, by elution using 1X SDS-PAGE sample buffer.

SDS-PAGE and western blotting
For fractionation assay, 1/10 Triton X-100-soluble and total pellet fractions were dissolved in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 1% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated on a 95°C heater for 5 min. Proteins were resolved on SDS-PAGE and transferred to Nitrocellulose Membrane (Bio-rad). For western blots assay with enhanced chemiluminescence (ECL) solutions (GE Healthcare), membranes were blocked for 1 h in TBST containing 5% non-fat milk, followed by incubation with primary antibodies at 4°C overnight. The blots were washed three times (10 min each) in TBST. After incubation with horseradish peroxide-coupled secondary antibodies and three washes with TBST, immunoblots were developed using ECL on films. For western blots assay using the Odyssey Imaging System (LI-COR Biosciences), membranes were blocked for 1 h in TBS instead of TBST containing 5% non-fat milk. After incubation with proper primary antibodies at 4°C overnight and wash with Tween 20 mixed Tris-buffered saline (TBST), three blots were incubated with fluorescein-labeled secondary antibodies (LI-COR Biosciences) for 1 h at room temperature in the dark. Membranes were washed four times for 10 min each at room temperature in PBST and one extra time for 10 min in PBS with gentle shaking in the dark. Data were acquired on an Odyssey Imager (LI-COR Biosciences).

Phosphatase treatment of cell lysates
Cells were lysed in a phospatase inhibitor-free lysis buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 50 mM KCl, 10 mM MgCl2, 1 mM DTT, protease inhibitor cocktail with 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A) containing 1% Triton X-100, 10 μl of calf intestinal alkaline phosphatase (New England BioLabs) was added to 500 μl lysates, followed by incubation at 37°C for 30 min.

In vitro phosphorylation and binding of BAG3 proteins
Phosphorylation of recombinant human BAG3 proteins (ProSpec) was carried out at 30°C for 1 h in 100 μl reactions containing 1 μg of BAG3, 20 μl of whole-cell extract from tsA201 cells, 20 mM Tris-HCl (pH 7.5), 120 mM NaCl, 25 mM NaF, 25 mM KCl, 10 mM EDTA, 10 mM MgCl2 and 2 μM ATP. The mixtures were then added to 1 μg of recombinant 14-3-3γ proteins (ProSpec), and 14-3-3 immunoprecipitation and western blot analysis were carried out as described above. For in vitro binding assays, 30 μl of bacterially expressed GST-mDIC fusion proteins coupled to glutathione-agarose beads was added to the mixture and incubated overnight at 4°C. Proteins bound to the beads were subjected to western blotting with specific antibodies.

Immunocytochemistry and confocal microscopy
Cells were grown on Poly-L-lysine-coated glass coverslips, fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized and blocked in a PBS solution containing 0.1% Triton (PBST) and 1% normal goat serum for 30 min at room temperature. They were then incubated in the same solution containing appropriate primary antibodies at 4°C overnight. The next day, sections were washed with PBST 3×5 minutes, followed by incubation with Alexa Fluor®
647 donkey anti-rabbit or anti-mouse secondary antibodies (Invitrogen) for 2 h at room temperature. After several washes with PBS and PBS, the coverslips were counterstained with 5 μg/ml DAPI (Sigma-Aldrich, St. Louis, MO) in PBS for 5 min, washed in PBS for 5 min, and mounted with Vectorshied to retard fluorescence fading. Cells were imaged on a Leica TCS SP2 SE laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) using a 63× objective at 1024×1024 resolution. Serial stack images were taken at 0.1 μm steps and all images were acquired by sequential scanning.

**Yeast strains, cytological and protein techniques**

Wild-type (WT) and bhm1Δ yeast strains are isogenic to Y300, a W303 derivative. A PΔαFLAG-Hht103QP-GFP fragment was integrated into yeast genome, so that the expression of Huntington disease gene with 103 poly-glutamine (Htt103QP) was induced after growth in galactose medium. To express the Drosophila 14-3-3α protein in yeast cells, we constructed the plasmid by inserting the cdNA of WT D14-3-3α or dimerization-deficient mutant D14-3-3Δ (MM14-3-3Δ) into a yeast expression plasmid under the control of yeast BMH1 promoter. Cells that express Htt103QP-GFP proteins were fixed with 3.7% formaldehyde for 5 min at room temperature and then resuspended in 1×PBS buffer for fluorescence microscopy. To analyze the Htt103QP protein levels, cells were resuspended in RAPI buffer (25 mM Tris pH 7.5, 10 mM EDTA, 150 mM NaCl and 0.05% Tween-20) supplemented with protease inhibitors, and then lysed by glass bead using a bead-beater. The lysates were centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant and pellet fractions were used for SDS-PAGE and western blot analysis using anti-FLAG antibody.

**RNA interference and rescue assay**

A549 HDAC6Δ-knockdown and tsA201 cells were transfected at 30–50% confluency using Lipofectamine RNAmax (Invitrogen), according to the manufacturer’s instructions, with 150 nM siRNAs targeting 14-3-3y (sc-29582, Santa Cruz), 14-3-3ε (sc-156019) or BAG3 (sc-72602, Santa Cruz). In the rescue assays, silent mutations (C1227A, T1230C, A1233C, G1341A, G1444A, T1347C) were introduced into cdNA of both wild-type and S136A/S173A BAG3 to make them resistant to BAG3 siRNA. They were then reintroduced into the cells that had been transfected with BAG3 siRNA 24 h earlier.

**Quantification of aggresome-containing cells**

For quantification analysis, five fields of each sample were randomly selected and over 100 cells were present in each field. The percentage of cells containing aggresomes was counted and averaged.

**Statistical analysis**

Student’s t-test was conducted for statistical analysis of quantitative data (expressed as mean ± s.e.m.). The value of * P<0.05 was considered a statistically significant difference.

**Acknowledgements**

We thank Drs Elizabeth S. Sztul and Jianhua Zhang (University of Alabama at Birmingham) for providing us the GFP250 and α-Syn cdNA constructs, Dr Ron R. Kopito (Stanford University) for the GFPFCTRFAS508 construct, Drs Qian Cai (Rutgers University) and Zu-hang Sheng (NIH) for the GFP-mDIC constructs, and Dr Yi Qiu (University of Florida) for providing us with the HDAC6 constructs and knockdown cell line. We also thank Ruth Didier of the Confocal (University of Florida) for providing us with the HDAC6 constructs and Dr Ron R. Kopito (Stanford University) for the GFP-mDIC constructs.

**Funding**

This work was supported by the National Institutes of Health [grant number NS0355 to Y.Z.Y.]. Deposited in PMC for release after 12 months.

**Supplementary material available online at**


Fig. S1. 14-3-3 enhances the accumulation of α-Syn-EGFPΔ155 in the insoluble fraction. α-SynEGFPΔ155 was transfected into tsA201 cells together with either 14-3-3γHA or pSCM138. Followed by a treatment of either ALLN (A) or DMSO (D) for 24 hs, whole cell lysates were separated into Triton-X100 soluble (supernatant) and insoluble (pellet) fractions, and samples from both fractions were analyzed by western blotting with an anti-α-Syn antibody (lanes 3-10). Lysates of cells transfected with the full-length α-SynEGFP were shown in lanes 1 and 2. Raw Odyssey Images are shown to depict the Benchmark™ protein ladder (left lane).
Fig. S2. Golgi positioning is not affected by 14-3-3 inhibition. Representative confocal images of CHO cells transfected with either the 14-3-3 binding antagonist pSCM138 (a) or its inactive control, pSCM174 (d). Cells were immunostained with Giantin antibodies to visualize Golgi apparatus (b and e; red), Golgi organization in pSCM138-transfected cells is similar to that in pSCM174-transfected or non-transfected cells. Scale bar, 10 μm.