# Huntingtin toxicity is ameliorated by BAG1 through modulation of its aggregation, degradation and subcellular distribution

**PhD Thesis**

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**submitted by**

Kamila Sroka

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Declaration

This thesis has been written independently and with no other sources and aids than quoted.

Kamila Sroka
Göttingen, March 2008

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**Contents**

# 1. INTRODUCTION

## 1.1 Huntington's disease

Huntington's disease (HD) is a dominantly inherited neurological disorder in which involuntary movements are accompanied by personality changes and dementia. It bears the name of the American physician George Huntington who first described it in 1872 (Huntington, 1872). The disease is often referred to as Huntington's chorea, from the Greek word *choreia* which means *dance*, due to dance-like uncontrollable movements that are its most remarkable symptoms. It manifests itself usually in midlife and progresses with choreiform movements, psychiatric dysfunction, dementia and weight loss (Folstein et al., 1986; Craufurd et al., 2001). There is no casual treatment available, and patients die about 10 to 20 years after disease onset. Brain autopsy reveals cell loss in the striatum, in particular of the medium spiny GABAergic neurons (Reiner et al., 1988), although other brain regions like cortex and hypothalamus are affected as well (Vonsattel et al., 1985; Petersen et al., 2005). Loss of neurons in the striatum explains the motor component of HD since it is a part of the basal ganglia circuit that regulates cortically initiated voluntary movements. Psychiatric and cognitive impairment most likely results from the dysfunction and loss of cortical neurons, as weight loss could be explained by degeneration of orexin neurons in the hypothalamus (Petersen and Bjorkqvist, 2006) and high metabolic turnover. In more advanced stages of HD, neuronal cell death is observed in other brain regions such as globus pallidus, subthalamic nuclei, substantia nigra, cerebellum and thalamus. At that stage the symptoms often change dramatically as patients can become rigid and akinetic.

## 1.2. Wild type huntingtin function

It took nearly ten years until the Huntington's Disease Collaborative Research Group was able to pinpoint the mutation responsible for HD. It lies in the *IT15* (interesting transcript 15) gene which contains 67 exons, is localized on chromosome 4p16.3 and codes for a 350 kDa protein called huntingtin. The mutation consists of an expansion of a CAG tract within exon 1 of the gene that encodes a polyglutamine stretch in the N-terminus of huntingtin (Huntington's Disease Collaborative Research Group, 1993). Wild type human huntingtin contains 6 to 35 glutamines in the stretch (Kremer et al., 1994). When the number is 36 or greater, it is believed to result in a toxic gain of function of the protein leading to disease. Interestingly, the length of the polyglutamine expansion is inversely proportional to the age of onset of HD (Andrew et al., 1993; Snell et al., 1993). It is worth noting that expanded CAG repeats are also found in at least eight other neurodegenerative diseases such as spinocerebellar ataxias 1, 2, 3, 6, 7 and 17, spinobulbar muscular atrophy and dentatorubral pallidoluysian atrophy, classified together as polyglutamine diseases. Although they affect different brain regions, and the disease-specific mutation is found in functionally and structurally unrelated proteins, some features of the pathological mechanism seem to be common (Margolis and Ross, 2001).

It is not known what the function of the polyglutamine stretch in huntingtin is. Evolutionary it first appeared in fishes that have 4 glutamines and was maintained in huntingtin homologues of all vertebrates suggesting that it may confer some advantageous properties to the protein (Baxendale et al., 1995; Cattaneo et al., 2005). Polyglutamine-rich domains are often found in transcription factors (for example TFIID, Sp1, homeobox protein of Drosophila), and it is speculated that they can act as "polar zippers", promoting interactions between proteins and enhancing transcription (Perutz et al., 1994).

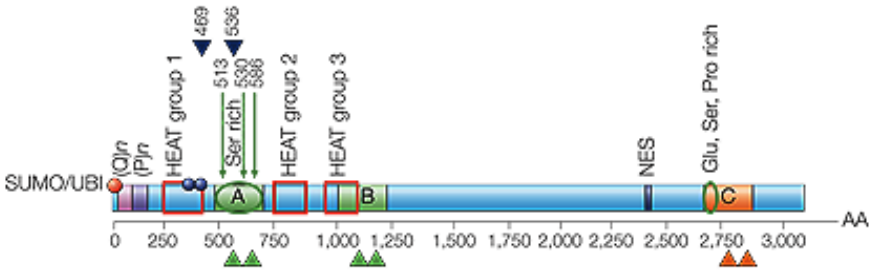



Fig. 1.1 A schematic illustration of huntingtin amino acid sequence.
At the N-terminus, starting at amino acid 18, is the polyglutamine (Q)n tract, which is 6-35 glutamines long in wild type protein and expanded above 36 repeats in the mutant form. The polyQ region is followed by a polyproline-rich region (P)n, which probably helps to keep the protein soluble (Steffan et al., 2004). Downstream are the so-called HEAT (**H**untingtin, **E**longation factor 3, protein phosphatase 2**A**, **T**OR1) repeats (red squares), which are tandemly repeated, 37-47 amino acid long domains that form rod-like helical structures which are involved in protein-protein interactions (Andrade and Bork, 1995). A nuclear export signal (NES) is localized in the C-terminus of the protein (Xia et al., 2003). Green arrows indicate the caspase cleavage sites and their amino acid positions, and blue arrowheads the calpain cleavage sites and their amino acid positions. Green and orange arrowheads point to the approximate amino acid regions for protease cleavage. The red circle at the N-terminus indicates the ubiquitin and SUMO modification site. The blue circles show the phosphorylation sites (serine 421 and serine 434). The glutamic acid (Glu)-, serine (Ser)- and proline (Pro)-rich regions are indicated. Modified from Cattaneo et al., 2005.

As far as the function of huntingtin as a whole is concerned, it remains unresolved as well. Huntingtin is a large protein of 350 kDa with no homology to other proteins (Fig. 1.1). It is completely soluble and was found in many subcellular compartments what further complicates the search for its function. It is mainly distributed diffusely throughout the cytosol but was also detected in neurites, synapses as well as the nucleus and associated with different cellular structures like the ER, Golgi, microtubules, clathrin-coated vesicles, synaptic vesicles, plasma membrane and mitochondria (DiFiglia et al., 1995; Gutekunst et al., 1995; Trottier et al., 1995; De Rooij et al., 1996; Kegel et al., 2002; Kegel et al., 2005). Huntingtin is expressed ubiquitously with the highest levels in the CNS and testes (Li et al., 1993; Sharp et al., 1995). Intriguingly, it appears to have a dual function, one during embryonic development and another one postnatally (Reiner et al., 2003). This view is based on studies on huntingtin

knock-out mice that are embryonically lethal at day 7.5 due to gastrulation defects (Nasir et al., 1995). Huntingtin is also crucial for the CNS formation as well as establishment and maintenance of neuronal identity, particularly in the cortex and striatum (White et al., 1997; Metzler et al., 1999; Metzler et al., 2000; Reiner et al., 2001). Interestingly, embryonic lethality can be completely rescued by over-expression of the mutant form of the protein, meaning that the mutation becomes pathogenic only in the adulthood (Leavitt et al., 2001; Van Raamsdonk et al., 2005). On the other hand, conditional knock-out of the gene in the forebrain and testes of adult mice leads to neurodegeneration and sterility (Dragatsis et al., 2000). These two different (and potentially multiple) huntingtin functions could be explained by different interaction partners. In screens, huntingtin was found to associate with proteins involved in clathrin-mediated endocytosis, apoptosis, vesicle transport, cell signaling, morphogenesis and transcriptional regulation, suggesting its importance for these processes (Borrell-Pages et al., 2006).

Indeed, huntingtin was found to be antiapoptotic in the nervous system. In particular, it is protective against ischemia (Zhang et al., 2003), excitotoxic insult (Leavitt et al., 2006) and mutant huntingtin toxicity *in vivo* (Leavitt et al., 2001). Studies of the underlying mechanisms showed that huntingtin inhibits the processing of caspase 9 (Rigamonti et al., 2001) and prevents the formation of the proapoptotic complex between HIP1 and HIPPI (Gervais et al., 2002).

Huntingtin stimulates transcription of neuronal genes by sequestering RE1-silencing transcription factor (REST, also known as neuronal restrictive silencing factor, NRSF) (Zuccato et al., 2007). The most studied example of such a gene is *Bdnf* (brain derived neurotrophic factor), coding for a neurotrophin produced in the cortex and retrogradely transported to the striatum, serving as a survival cue for striatal neurons and as a signal to maintain cortico-striatal synapses (Zuccato et al., 2001).

Huntingtin has also been implicated in fast axonal transport (Szebenyi et al., 2003) and transport of BDNF (Gauthier et al., 2004). There are also reports that show a relevance of huntingtin at the synapse where it interacts with PSD-95, affecting the function of NMDA receptors (Sun et al., 2001).

## 1.3. Mutant huntingtin

In contrast to wild type huntingtin, much more effort was put into studies of the mutant form of huntingtin, focusing mainly on the N-terminal part of the protein that contains the polyglutamine stretch and is sufficient to cause neurodegeneration *in vivo* (Mangiarini et al., 1996). Besides the size of full-length huntingtin, the reason why most laboratories use only the amino-terminal part of the protein is that truncated N-terminal fragments were found to accumulate and to be toxic in HD models (Mende-Mueller et al., 2001). Years of research showed that the mutation has a deleterious effect on a plethora of cellular processes which will be discussed below (Fig. 1.2).

### 1.3.1 Cleavage and nuclear translocation

The N-terminal part of huntingtin contains recognition sites for calpain and caspases 1, 3, 6, 7, and 8 (Mende-Mueller et al., 2001; Lunkes et al., 2002; Gafni et al., 2004; Hermel et al., 2004) (Fig. 1.1). Both wild type and mutant huntingtin are processed by those proteases although the latter to a greater extent what is believed leads to toxicity. It was recently discovered that not all cleavage products share identical properties. The fragment generated by caspase 6 cleavage leads to neurodegeneration, whereas the one generated by caspase 3 does not (Graham et al., 2006). The exact sequence and relevance of the various cleavage events is not clear yet; nonetheless, it is well established that the truncated fragment translocates to the nucleus. This seems to constitute the key event in HD pathophysiology (Saudou et al., 1998; Peters et al., 1999) since inhibition of nuclear translocation prevents striatal cell death.

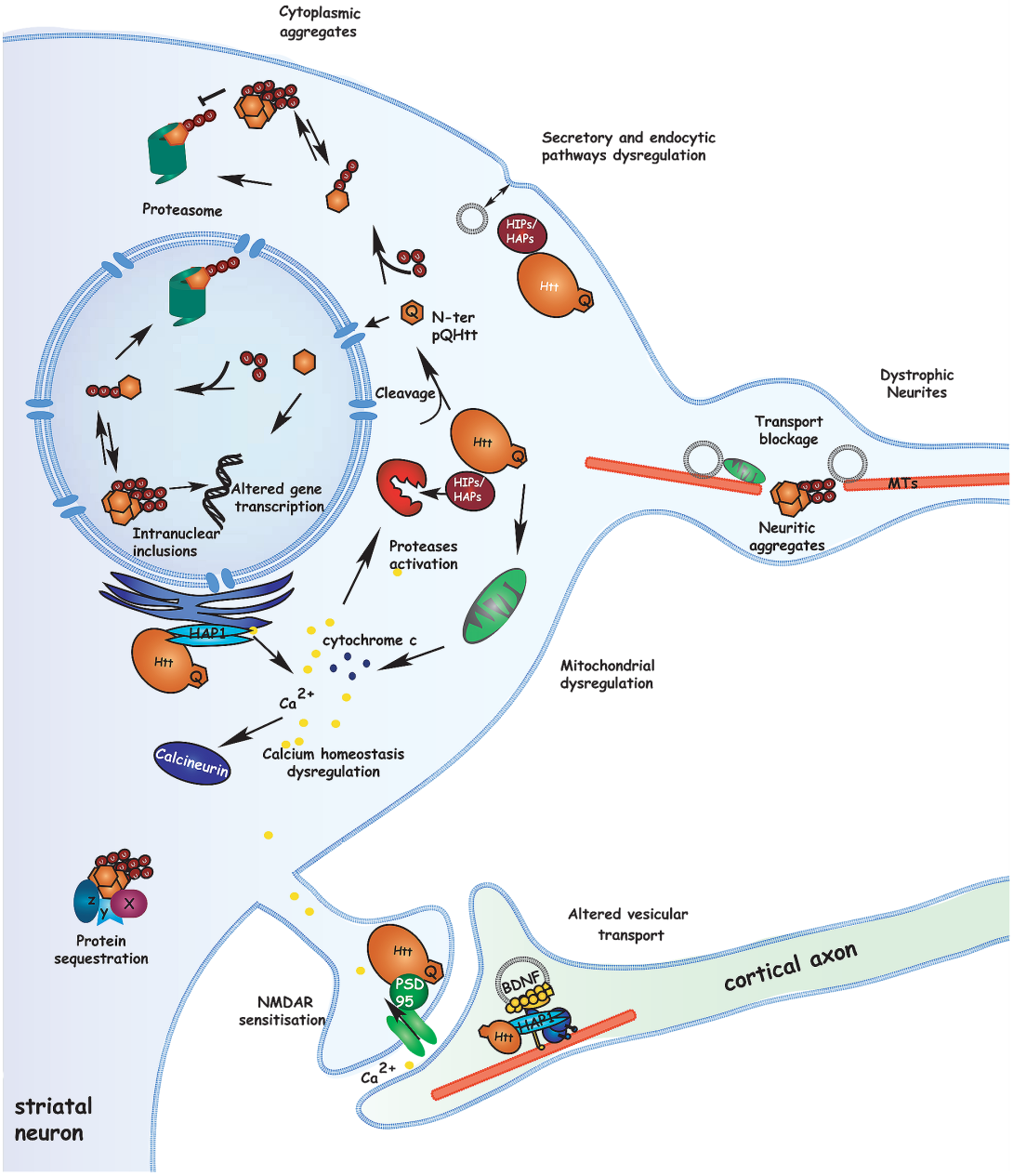



Fig. 1.2 Mutant huntingtin disrupts an array of cellular processes.
The N-terminal part of huntingtin, which contains the polyQ expansion, is cleaved off by proteases. The fragment translocates to the nucleus, where it disrupts transcription and induces cell death. Furthermore, the fragments aggregate forming inclusion bodies in the cytosol and in the nucleus. The inclusions contain ubiquitin, proteasomal components and chaperones. Mutant huntingtin leads to an impairment of the ubiquitin-proteasome system. It deregulates mitochondrial function and calcium homeostasis. It alters vesicular transport and recycling, while inclusions in neurites may physically block transport. Defect in BDNF transport increases vulnerability of striatal neurons to cell death. Htt, Huntingtin; HIPs, huntingtin-interacting proteins; HAPs, huntingtin-associated proteins. From Borrell-Pades et al., 2006.

### 1.3.2. Disruption of transcription

The mechanism of action of the toxic fragment in the nucleus probably involves its interactions with transcription factors, many of which also have polyglutamine domains (Gerber et al., 1994). Mutant huntingtin binds to p53, CREB-binding protein, TBP and Sp1/TAFII130 transcription factors (Nucifora et al., 2001; Wyttenbach et al., 2001; Dunah et al., 2002; Schaffar et al., 2004; Bae et al., 2005; Zhai et al., 2005). Microarray experiments showed aberrant transcription of many genes, including those coding for neurotransmitter receptors and their corresponding second messenger system components (Luthi-Carter et al., 2000; Luthi-Carter et al., 2002; Sipione et al., 2002; Desplats et al., 2006). Moreover, mutant huntingtin no longer binds to REST/NRSF what leads to silencing of several neuronal genes, including the *Bdnf* gene (Zuccato et al., 2003). This could contribute to selective neuronal vulnerability in HD as striatal neurons strongly depend on cortical BDNF signals (Altar et al., 1997; Baquet et al., 2004).

### 1.3.3. Aggregation

Mutant huntingtin, as many other mutant proteins implicated in neurodegeneration, forms inclusion bodies in the cytosol and in the nucleus (called neuronal intranuclear inclusions, NIIs) composed of N-terminal fragments of the protein (DiFiglia et al., 1997; Becher et al., 1998). These inclusions (also referred to as aggregates) were characterized to have an ordered fibrillar amyloid-like structure. Polyglutamine chains form β-hairpin structures (also called “polar zippers”) held together by hydrogen bonds between the main-chain and side-chain amides (Perutz et al., 1994). The relationship between inclusion bodies and HD pathophysiology is a matter of debate. It is speculated that they might interfere with normal cell function by sequestering components of the chaperone system, the ubiquitin-proteasome system (UPS) and transcription factors (McCampbell et al., 2000; Jana et al.,

2001; Nucifora et al., 2001; Hansson et al., 2003; Hay et al., 2004). Due to their large size, aggregates may also block axonal transport (Gunawardena et al., 2003), and they are found in dystrophic neurites in HD brains (DiFiglia et al., 1997; Maat-Schieman et al., 1999). On the other hand, their occurrence does not correlate with cell death (Saudou et al., 1998; Kim et al., 1999; Slow et al., 2005), and it was recently shown that cells which develop aggregates have in fact higher chances of survival (Arrasate et al., 2004). The inclusions might then have a beneficial function because they can sequester soluble mono- or oligomeric toxic fragments of mutant huntingtin and prevent them from interacting with other proteins like transcription factors. Nevertheless, the inclusions are predominantly seen in those neurons that are most affected by the disease (DiFiglia et al., 1997). In addition, the most popular mouse model of HD, the R6/2 mouse line, exhibits high amounts of huntingtin aggregation and neuronal dysfunction closely resembling HD but almost no striatal cell death (Mangiarini et al., 1996; Li et al., 2005). Importantly, the formation of inclusion bodies precedes the onset of symptoms in this model (Davies et al., 1997). This points out to the possibility that although aggregates are not directly causing cell death they do contribute to the loss of neuronal function.

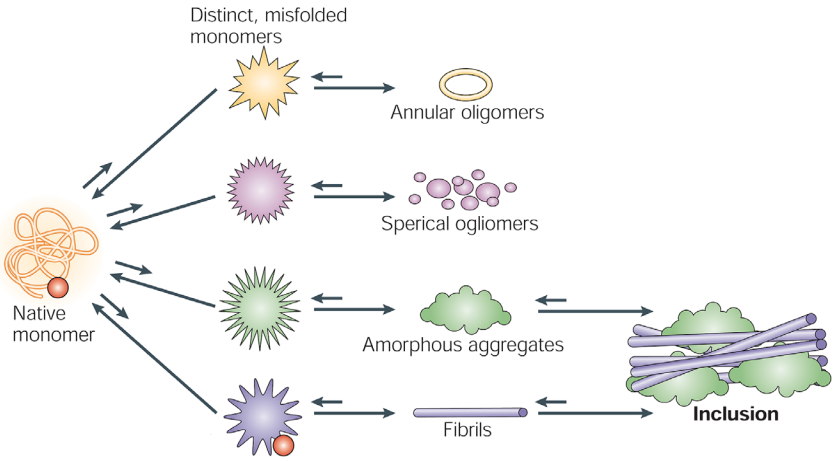



Fig. 1.3 A model of the assembly of polyQ expansion proteins into amyloid-like fibrils.
A native monomer can change its conformation into a set of distinct misfolded monomers. In order to misfold, the protein has to overcome a high-energy barrier depicted by the rightward-pointing arrows. Each of the misfolded monomers can give rise to higher order species, like annular oligomers, spherical oligomers, amorphous aggregates or fibrils. It is believed that amorphous aggregates, fibrils and inclusions are protective, while oligomers are toxic. Modified from Muchowski and Wacker, 2005.

It has to be noted, however, that the aggregation process is quite complex, and it was shown that there are various stages with oligomers and aggregate intermediates of different properties (Wacker et al., 2004) (Fig. 1.3). Most studies concentrate on the last step of macroscopic aggregates that are possible to visualize under a light microscope and do not take into account smaller microaggregates, oligomeric species, subtle morphological changes or changes in biochemical properties that are probably more relevant to toxicity.

### 1.3.4. The ubiquitin-proteasome pathway

All cellular proteins are continually being synthesized and degraded as part of normal cell function. To ensure cell viability, damaged or mutated proteins have to be removed. The ubiquitin-proteasome system (UPS) is a complex multi-enzymatic machinery responsible for highly selective intracellular protein degradation (Ciechanover, 2005) (Fig. 1.4). To be degraded, a protein needs to be marked with a covalently attached chain consisting of multiple moieties of a protein ubiquitin, linked through lysine 48. The machinery responsible for ubiquitin-tagging consists of three types of enzymes: E1, the ubiquitin-activating enzyme, E2, the ubiquitin-conjugating enzyme and E3, the substrate-specific ubiquitin ligase. Once the target protein is tagged with a polyubiquitin chain, it is recognized as a proteasomal substrate and degraded into smaller peptides. The 26S proteasome is a ~1,5 MDa enzyme composed of two subcomplexes. The 20S barrel shaped core particle has six proteolytic sites: two of them preferentially cleave after hydrophobic residues, two after basic ones and two after acidic ones (Coux et al., 1996). The 19S regulatory particle is located at both ends of the 20S particle. It recognizes polyubiquitin chains, unfolds substrates in an ATP-dependent way and inserts them to the core particle for degradation.

Mutant huntingtin inclusions stain positively for ubiquitin, proteasomal components and chaperones (Davies et al., 1997; Jana et al., 2000;

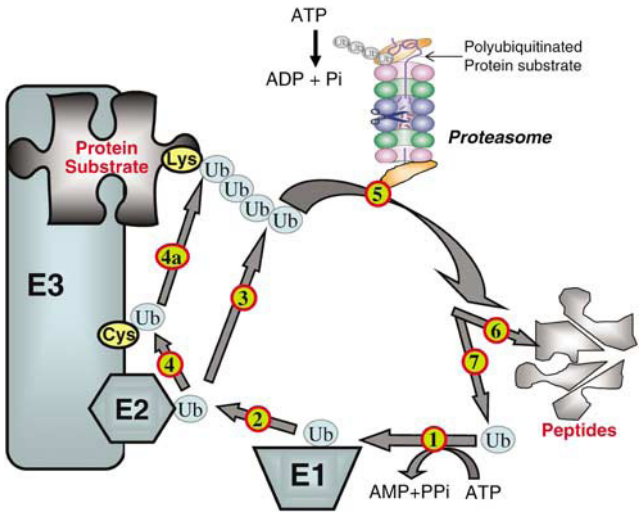



Fig. 1.4 The ubiquitin–proteasome system.
Ubiquitin is activated by the ubiquitin-activating enzyme, E1 (1) and transferred to a ubiquitin-conjugating enzyme (UBC), E2 (2). E2 transfers ubiquitin to the substrate protein bound to a ubiquitin ligase, E3. Ubiquitin ligases recognize substrates and confer specificity to the degradation machinery. If the E3 ligase belongs to the RING finger family, the transfer of ubiquitin is direct from E2 to the substrate protein (3). In case of the HECT type E3 ligases, ubiquitin is first transferred to the ligase and then conjugated to the substrate (4). Polyubiquitin chain serves as a recognition signal for the proteasome, which degrades the substrate protein into short peptides (5, 6). Ubiquitin is cleaved off by de-ubiquitinating enzymes (DUBs) and can be reused in another cycle of degradation (7). From Ciechanover, 2005.

Wyttenbach et al., 2000; Jana et al., 2001; Waelter et al., 2001). This led to the hypothesis that mutant huntingtin has a different conformation than the wild type protein and is recognized by cellular chaperones as misfolded. Failure to correct its structure targets the mutant protein for degradation via the UPS. Localization of the UPS components to huntingtin aggregates could indicate that they are specifically recruited and that proteolysis occurs at high rates (Chen et al., 2008). Alternatively, it could mean that the degradation attempt was unsuccessful, and the degradation components became trapped in the aggregates. Long glutamine stretches are in fact considered to be a “difficult” substrate for the proteasome (Jana et al., 2001; Venkatraman et al., 2004) and may block its function. It was shown that eukaryotic proteasomes cannot digest polyglutamine stretches *in vitro* and that they release them for degradation by the puromycin-sensitive aminopeptidase (Bhutani et al., 2007). The idea of the UPS impairment by expanded polyglutamine proteins received much

attention in recent years. For instance, pharmacological inhibitors of the proteasome increase the amount of mutant huntingtin aggregation (Jana et al., 2001; Waelter et al., 2001). In addition, the proteasome was shown to be impaired in experiments using fluorescent sensors of its function (Bence et al., 2001; Verhoef et al., 2002). Furthermore, accumulation of Lys-48 linked ubiquitin chains, as well as Lys-11 and Lys-63 chains normally not associated with protein degradation, was observed in animal models of HD (Bennett et al., 2007). Moreover, expression of proteins that stimulate proteasomal degradation, like E3 ligases Hrd1, E6-AP and CHIP or proteasome activator PA28, protects from mutant huntingtin toxicity (Jana et al., 2005; Al-Ramahi et al., 2006; Seo et al., 2007; Yang et al., 2007; Mishra et al., 2008). In a conditional HD mouse model switching off the mutant gene leads to a complete clearance of inclusion bodies (and reversal of motor symptoms) in a proteasome-dependent manner (Yamamoto et al., 2000; Martin-Aparicio et al., 2001). This signifies that the proteasome has the intrinsic capability of removing mutant huntingtin and suggests a potential therapeutic route of enhancing its activity.

### 1.3.5. Autophagy

Another intracellular degradation pathway, which only recently gained interest with regard to its importance for disease, is autophagy (specifically macroautophagy). It involves the formation of a double membrane autophagosome around a portion of the cytoplasm that later fuses with a lysosome. The contents of the autophagic vacuole are then degraded by lysosomal hydrolases (Fig. 1.5). The process of autophagosomal membrane expansion involves Atg (autophagy-related genes) proteins that bear some resemblance to the components of the ubiquitin chain formation machinery (Rubinsztein et al., 2007). The difference between the two degradation routes is that the UPS is more specific and targets short-lived nuclear and cytosolic proteins, while autophagy, besides membrane-bound proteins and whole organelles, removes all proteins that happen to be in the portion of the

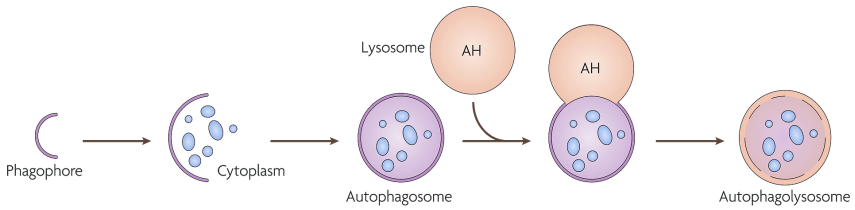



Fig. 1.5 A schematic illustration of macroautophagy.
A phagophore membrane, of so far unknown origin, expands, sequestering cytoplasm and eventually forming a double-membrane autophagosome. Subsequently, the autophagosome fuses with a lysosome, which contains acidic hydrolases (AH). The fused compartment where the contents of the autophagosome are degraded is called an autophagolysosome or an autolysosome. Nutrients generated by macromolecular autophagic degradation are important for cell survival during starvation. Autophagy also degrades microbial pathogens as part of the immune response. Recently, it has become clear that autophagic degradation is crucial for the function of the nervous system and has implications in several neurodegenerative diseases. Modified from Rubinsztein et al., 2007.

cytoplasm engulfed by the autophagic membrane. In addition, autophagy can remove folded substrates as opposed to proteasomes which require that a protein is unfolded before it is inserted into the narrow barrel of the proteasomal core.

Mutant huntingtin was observed in association with multivesicular bodies (organelles involved in protein degradation) in brains of HD patients (Sapp et al., 1997) and autophagosomes in cell culture (Kegel et al., 2000). Furthermore, the number of autophagosomes is elevated in HD models (Petersen et al., 2001; Nagata et al., 2004). This could reflect either an induction of autophagy or a decrease in fusion events between autophagosomes and lysosomes. Aggregates of mutant huntingtin sequester mTOR (mammalian target of rapamycin) protein, which is an inhibitor of autophagy. Thus, inclusion body formation could serve as a protective mechanism stimulating autophagy that in turn removes the aggregation-prone protein. The macroscopic aggregates themselves, however, are too big to be removed by means of autophagy, which probably targets soluble species and oligomers (Rubinsztein et al.,

2005). It was shown that stimulation of autophagy reduces the levels of mutant huntingtin, decreases the amount of aggregation and has a neuroprotective effect (Ravikumar et al., 2002; Ravikumar et al., 2004; Iwata et al., 2005; Shibata et al., 2006). Interestingly, neuron-specific knock-out of autophagy related genes in mice results in a progressive neurodegenerative phenotype with motor abnormalities and formation of inclusion bodies containing ubiquitinated proteins what further underscores the importance of autophagy in the brain (Hara et al., 2006; Komatsu et al., 2006).

### 1.3.6. Mitochondrial dysfunction

Defects in energy metabolism were first indicated by the fact that HD patients suffer from severe weight loss despite increased caloric intake. Subsequent studies indeed revealed aberrations in mitochondrial function. Lymphoblasts from HD patients show reduced mitochondrial membrane potential and $Ca^{++}$ uptake (Panov et al., 2002) as well as profound morphological abnormalities of mitochondria (Squitieri et al., 2006). Mutant huntingtin was found to bind to the outer mitochondrial membrane in cell culture (Choo et al., 2004). It significantly decreased $Ca^{++}$ threshold necessary to trigger mitochondrial permeability transition pore opening, which was accompanied by a release of cytochrome c (a key event in the apoptotic cascade (Zamzami and Kroemer, 2001)). Defects in aconitase, complex II and III of the respiratory chain as well as abnormalities in mitochondrial structure were observed in biopsies and post mortem brain samples of HD patients (Goebel et al., 1978; Browne et al., 1997; Tabrizi et al., 1999). Mutant huntingtin also leads to decreased mitochondrial ATP levels and mitochondrial ADP-uptake (Seong et al., 2005). Moreover, exposure to 3-nitropropionic acid, an inhibitor of complex II leads to symptoms resembling those of HD (Beal et al., 1993; Ludolph and Munch, 1999). Interestingly, knock-out mice of PGC-1α, a gene crucial for mitochondria biogenesis and defense mechanisms against reactive oxygen species, develop a neurodegenerative phenotype reminiscent of HD with motor impairment and cell loss in the striatum (Lin et al., 2004).

### 1.3.7. Signaling pathways implicated in HD

Several signal transduction pathways are modified in response to mutant huntingtin. Among them, activation of mitogen-activated protein kinase (MAPK)-signaling was observed. MAP kinase pathways are highly conserved cascades of protein kinases, activated by a range of growth factors and other stimuli. They regulate cell growth, differentiation and apoptosis (Cooper, 2000; Johnson and Lapadat, 2002). Generally, activation of the extracellular signal-regulated kinase (ERK) and the PI3K-Akt signaling pathways promotes cell survival, whereas activation of the stress-activated protein kinases (SAPK's): c-Jun N-terminal kinases (JNK's) and the p38 MAP kinase (p38 MAPK) leads to cell death (Fig. 1.6).

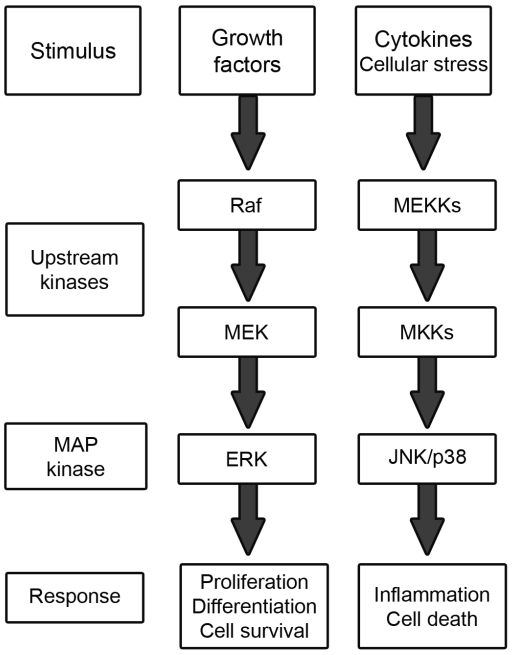



Fig. 1.6 A schematic illustration of the MAP kinase pathway.
Extracellular stimuli initiate a cascade of phosphorylation events in which upstream kinases act on downstream ones. The MAP kinases themselves phosphorylate many cellular targets, including transcription factors, affecting a variety of cellular responses. Activation of ERK kinase leads to cell growth and differentiation. Activation of p38 and JNK kinases results in inflammatory response or cell death. ERK, extracellular signal-regulated kinase, JNK, c-Jun N-terminal kinase, MAP mitogen-activated protein, MEK, MAP/ERK kinase, MEKK, MEK kinase, MKK, MAP kinase kinase. Modified from Cooper, 2000.

Mutant huntingtin activates the proapoptotic JNK kinase in several models of HD (Liu, 1998; Merienne et al., 2003; Apostol et al., 2006). Furthermore, the Akt pathway was shown to be altered in HD and activation of ERK/Akt prosurvival kinases was found to protect from mutant huntingtin toxicity (Humbert et al., 2002; Colin et al., 2005; Varma et al., 2007). Akt kinase phosphorylates mutant

huntingtin at serine 421, reducing its toxicity (Humbert et al., 2002; Warby et al., 2005). Another kinase acting on huntingtin is Cdk5 which phosphorylates it at serine 434 reducing its cleavage, aggregation and toxicity (Luo et al., 2005).

## 1.4. Chaperone-mediated neuroprotection

In the crowded and highly-reactive cellular environment, proteins, which are not very stable at 37°C, are at permanent risk of spontaneous denaturation or abnormal chemical modification that could lead to aberrant interactions with other proteins (Ellis, 1997). To tackle such problems, organisms have developed a very conserved class of proteins called molecular chaperones. They correctly fold newly synthesized proteins, recognize and refold proteins which have lost their conformation, prevent aggregation and target unfolded proteins for degradation (Hartl and Hayer-Hartl, 2002). Together with the UPS, they constitute the cellular protein quality control system. In response to stressful stimuli (including but not limited to temperature elevation), many chaperones (heat shock proteins, Hsps) are induced or upregulated as part of the so-called heat shock response which serves to maintain cell function and viability. Chaperones act by binding to hydrophobic surfaces of other proteins thereby shielding them from the cellular milieu, preventing unwanted interactions and protecting them from the formation of off-pathway intermediates and aggregation (Bukau and Horwich, 1998; Wegele et al., 2004). Furthermore, chaperones recognize misfolded substrates and target them for proteasomal degradation (Lee et al., 1996; Bercovich et al., 1997). Proteins whose erroneous structure is not corrected tend to oligomerize and aggregate. Since aggregates are a pathologic hallmark feature of HD and other polyglutamine diseases, it appears that the protein quality control pathway is not working efficiently in those disorders. Therefore, stimulating chaperone activity was suggested to be another strategy with high therapeutic potential.

### 1.4.1. Hsp70 and Hsp40

Hsp70 and Hsp40, two chaperones involved in folding of nascent peptides, were given particularly high attention in polyglutamine diseases. Both are able to reduce the amount of polyQ aggregation and/or toxicity in cell culture (Cummings et al., 1998; Chai et al., 1999; Jana et al., 2000; Kobayashi et al., 2000). They interact with mutant huntingtin in a polyglutamine-length dependent manner and localize to inclusion bodies (Cummings et al., 1998; Stenoien et al., 1999; Jana et al., 2000). They were also shown to increase the degradation of mutant androgen receptor (which underlies spinobulbar muscular atrophy) by the proteasome (Bailey et al., 2002). Both chaperones mediate protection from polyQ-induced toxicity in *D. melanogaster* (Warrick et al., 1999; Chan et al., 2000; Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000), and Hsp70 significantly improves the phenotype in a mouse model of spinocerebellar ataxia 1 (Cummings et al., 2001), although without affecting the formation of inclusions. This was explained by *in vitro* studies which showed that Hsp70 together with Hsp40 facilitates the formation of detergent-soluble amorphous aggregates (as opposed to detergent-insoluble fibrillar ones) as seen by biochemical analysis and atomic force microscopy (Muchowski et al., 2000; Wacker et al., 2004). These two types of aggregates are indistinguishable by light microscopy but have very different biochemical properties. However, overexpression of Hsp70 in the R6/2 HD mouse model has only a minor effect on disease progression (Hansson et al., 2003; Hay et al., 2004).

### 1.4.2. Other chaperones

Expression of the yeast chaperone Hsp104 in *C. elegans* suppresses both aggregate formation and toxicity of expanded polyglutamine proteins (Satyal et al., 2000). A mammalian chaperone VCP/p97 (valosin-containing protein, a member of the AAA+ family of ATPase proteins), which is distantly related

to Hsp104, localizes to polyQ aggregates and modifies their formation (Hirabayashi et al., 2001; Boeddrich et al., 2006; Kobayashi et al., 2007). Paradoxically, both in case of Hsp104 and VCP/p97, chaperone knock-down has the same effect on aggregation as its excess. This means that they participate both in aggregate formation and solubilization, depending on the amount and balance with other molecular chaperones. A small chaperone Hsp27 was shown to suppress mutant huntingtin-induced reactive oxygen species formation and death in cell culture (Wyttenbach et al., 2002). However, double transgenic R6/2/Hsp27 mice again failed to show any improvement (Zourlidou et al., 2007). Two recent studies report that the cytosolic chaperonin (CCT)/TRiC reduces mutant huntingtin aggregation and promotes the formation of nontoxic oligomers (Behrends et al., 2006; Kitamura et al., 2006). Additionally, a disaccharide trehalose, which is a chemical chaperone in some species, was found to inhibit polyQ aggregation and lead to an amelioration of the phenotype in the R6/2 mouse model of HD (Attfield, 1987; Tanaka et al., 2004).

### 1.4.3. The relevance of molecular chaperones in neurodegeneration

The importance of chaperones for normal cell function is further underscored by a study showing that a dominant negative mutant of Hsp70 leads to a neurodegenerative phenotype even in the absence of neurodegeneration-related protein overexpression in *D. melanogaster* (Auluck et al., 2002). Furthermore, it was shown that the induction levels of Hsp70 in neurons of different brain structures correlate with their sensitivity to mutant huntingtin (Tagawa et al., 2007). Cortical and striatal neurons, which are most vulnerable in HD, displayed lower Hsp70 levels, while cerebellar granule neurons, which are spared in HD, upregulated their Hsp70 levels in response to mutant huntingtin. Moreover, expression of polyQ expansion proteins in *C. elegans* disrupts the global balance of protein folding quality control (Gidalevitz et al., 2006). Unbiased genetic screens for modifiers of polyQ toxicity and

aggregation in *S. cerevisiae*, *D. melanogaster* and *C. elegans* identified Hsp70 and Hsp40 homologues as the most potent suppressors (Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Willingham et al., 2003; Nollen et al., 2004).

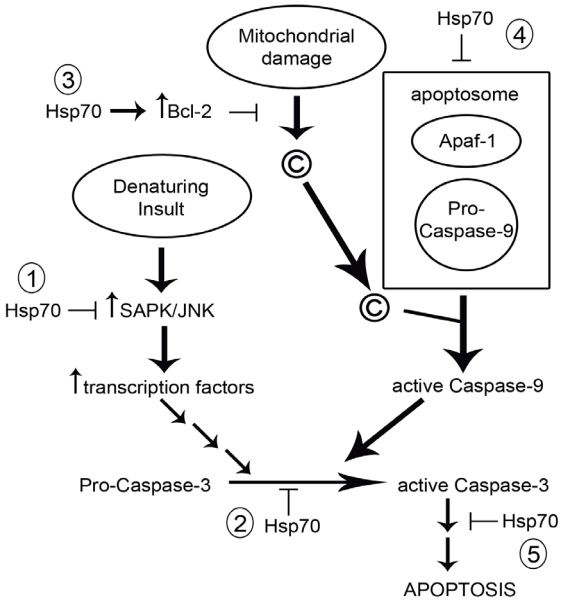



Fig. 1.7 Hsp70 can inhibit apoptosis in several ways.
(1) It inhibits the SAPK/JNK kinase. (2) It inhibits caspase 3 activation. (3) It increases the expression of the antiapoptotic Bcl-2. (4) It prevents the formation of the apoptosome. (5) It can also act downstream of caspase 3 activation. Modified from Eurekah Bioscience Collection, © 2000-2005 Landes Bioscience.

In addition to protection from unfolded proteins and stimulation of their degradation, chaperones can interfere with other detrimental processes that occur in HD, including apoptosis, oxidative stress and abnormal activation of signaling pathways (Fig. 1.7) (Zhou et al., 2001). They are, therefore, an interesting therapeutic target for HD and other neurodegenerative diseases. However, as discussed above, their mechanisms of action are still not fully understood and there are often conflicting data regarding chaperone-mediated neuroprotection. More studies are needed to be done in order to explain the mechanisms underlying different effects and contradicting results.

## 1.5. BAG1

BAG1 (Bcl-2-associated athanogene) is an antiapoptotic protein discovered in a screen for molecules that bind the anti-cell death protein Bcl-2 (Takayama et al., 1995). There are four different isoforms in humans (BAG1, BAG1-L –M and –S) and two in mice (BAG1-L and BAG1), originating from one mRNA by alternative translation initiation site (Packham et al., 1997; Takayama et al., 1998; Yang et al., 1998). Meanwhile, five more human BAG proteins were reported. They share a highly conserved C-terminal 110-124 amino acid long BAG domain but differ greatly in their N-terminal domains (Takayama et al., 1999; Doong et al., 2002).

BAG1 is particularly interesting due to its many interaction partners and its involvement in a wide array of cellular processes. Overexpression of BAG1 isoforms has been demonstrated to regulate apoptosis, protein degradation, proliferation, transcription, metastasis and cell motility (see table 1 for a list of its binding partners and functions). It acts as a nucleotide exchange factor for the Hsp70 family of molecular chaperones to which it binds through its BAG domain. It stimulates Hsp70 ATPase activity and unloading of the substrate (Hohfeld and Jentsch, 1997; Takayama et al., 1997; Zeiner et al., 1997; Bimston et al., 1998; Gassler et al., 2001; Sondermann et al., 2001). In an *in vitro* study using a luciferase-based assay to monitor the Hsp70 activity, it was found to negatively influence Hsp70 ability to refold luciferase after a heat shock (Nollen et al., 2000). However, recent studies show that BAG1 can also function as a stimulatory interaction partner of Hsp70 (Luders et al., 2000; Terada and Mori, 2000; Gassler et al., 2001; Liman et al., 2005). These discrepancies reflect the complexity of the protein folding machinery whose function depends on the cell type, conditions and the delicate balance between chaperones and their co-factors.

Another conserved domain shared between all isoforms of BAG1 is a ubiquitin-like motif in the N-terminus of the protein indicating its role in protein

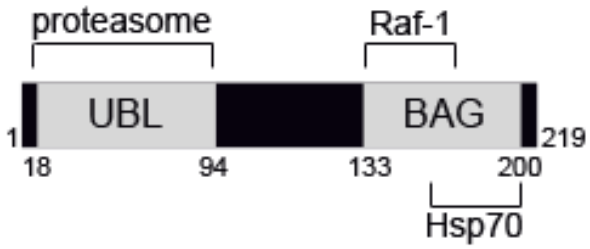



Fig. 1.8 The structure of the murine BAG1.
Mouse BAG1 (short isoform) is a 219 amino acid long protein. The BAG and the UBL (ubiquitin-like) domains are marked as grey boxes. Numbers correspond to the boundaries of these motifs. Binding sites of Hsp70, Raf-1 and the proteasome are indicated.

degradation (Fig. 1.8). BAG1 was found to bind to the 26S proteasome and to the E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein) (Luders et al., 2000; Alberti et al., 2002). It was shown that CHIP adds a Lys-11 linked ubiquitin chain to BAG1, what increases its association with the proteasome but does not lead to its degradation. BAG1 in turn recruits Hsc70/Hsp70 chaperones to the proteasome. It was demonstrated that BAG1 working in a complex with CHIP can enhance proteasomal degradation of the glucocorticoid hormone receptor (Demand et al., 2001) (Fig. 1.9).

BAG1 also binds and stimulates the serine/threonine protein kinase Raf-1, thus activating the MAP kinase-ERK pathway leading to cell growth and differentiation (Wang et al., 1996; Kermer et al., 2002) (Fig. 1.6). Binding to

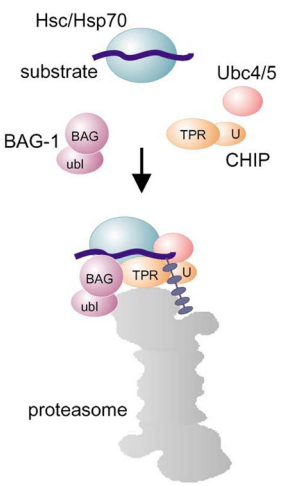



Fig. 1.9 A model of chaperone-mediated targeting of a substrate protein for degradation by the proteasome. The E3-ligase CHIP associates with Hsp70 and recruits an E2 ubiquitin-conjugating enzyme from the Ubc4/5 family to the complex. Together they ubiquitinate the Hsp70-bound substrate. BAG1 binds to Hsp70 through the BAG domain. It targets the whole complex to the proteasome by associating with it through its ubiquitin-like domain, thereby facilitating degradation of the substrate. From Demand et al., 2001.

Raf-1 is mediated by the second and third helices of the BAG domain, while binding to Hsp70 through the first and second helices. These two interactions of BAG1 are therefore mutually exclusive and it has been suggested that, since BAG1 cellular levels are lower than that of Hsp70 or Raf-1, they might be competing for BAG1 binding. Consequently, BAG1 could serve as a switch between cell growth and cellular stress response (Song et al., 2001). When cells are under stress, Hsp70 levels increase preventing BAG1 from interacting and stimulating Raf-1. This diminishes Raf-1 signaling and inhibits events like DNA synthesis leading to an arrest in cell cycle.

BAG1 is particularly important for the nervous system. It stimulates neuronal differentiation (Kermer et al., 2002) and its mouse knock-out is embryonically lethal due to massive apoptosis in the liver and in the nervous system (Gotz et al., 2005). It also protects from ischemic damage (Kermer et al., 2003) and various other apoptotic insults (Takayama et al., 1995; Schulz et al., 1997; Townsend et al., 2003; Townsend et al., 2004).

**Table 1** Interaction partners of BAG1. Plus sign means BAG1 has a stimulatory effect on the binding partner, minus sign indicates inhibitory effect.

| Binding partner | Effect | Isoform | Reference |
|---|---|---|---|
| Bcl-2 | + | BAG-1 | (Takayama et al., 1997) |
| Hsc70/Hsp70 | +/- | all isoforms | (Nollen et al., 2000; Terada and Mori, 2000; Gassler et al., 2001; Liman et al., 2005) |
| Proteasome | | BAG-1 | (Luders et al., 2000; Alberti et al., 2002; Elliott et al., 2007) |
| CHIP | + | BAG-1 | (Demand et al., 2001) |

| | | | |
|---|---|---|---|
| Siah1 | - | BAG-1 | (Matsuzawa et al., 1998) |
| Raf1 | + | BAG-1 | (Wang et al., 1996; Song et al., 2001) |
| Hepatocyte growth factor; platelet derived growth factor | + | BAG-1 | (Bardelli et al., 1996) |
| Glucocorticoid receptor | - | BAG-1L<br>BAG-1M | (Kullmann et al., 1998; Schneikert et al., 1999; Schmidt et al., 2003) |
| Androgen receptor | + | BAG-1L | (Froesch et al., 1998) |
| Retinoic acid receptor | - | BAG-1 | (Liu et al., 1998) |
| DNA (stimulation of transcription) | + | BAG-1L,<br>BAG-1M | (Zeiner et al., 1999; Niyaz et al., 2001; Takahashi et al., 2001) |

# 2. AIMS OF THE STUDY

The purpose of this project was to investigate the potential of BAG1 in ameliorating mutant huntingtin toxicity. BAG1 appears as an interesting therapeutic target in context of HD since it links many cellular pathways that are implicated in HD, like protein folding, the UPS, ERK signaling and the apoptotic machinery. Moreover, BAG1 can bind and inhibit Siah1, a p53-inducible proapoptotic protein (Matsuzawa et al., 1998), which was recently identified as being crucial for nuclear translocation of the mutant huntingtin fragment (Bae et al., 2006). Thus, we hypothesized that BAG1 may aid in refolding of mutant huntingtin, its degradation or inhibit its nuclear accumulation. In this study, we perform a detailed examination of BAG1 effects on the above mentioned processes as well as on mutant huntingtin toxicity in various *in vitro* and *in vivo* HD models.

# 3. MATERIALS AND METHODS

## 3.1 Chemicals

**Applichem***:* 2-Propanol, Chloroform, Glycine, Guanidine hydrochloride, Imidazole, Milk powder, Tris, Tween-20
**Biorad:** Precision Plus Protein dual color standard
**Calbiochem***:* Moviol, ProteoExtract Subcellular Proteome Extraction Kit
**Fluka:** p-coumaric acid
**Gibco:** OptiMEM
**GeReSo mbH:** Ethanol
**Invitrogen:** DNase I, Lipofectamine 2000, RNase H, SuperScript III Reverse Transcriptase
**Macherey-Nagel:** NucleoSpin Plasmid miniprep kit
**Merck***:* Ammonium peroxide, DAPI, Hydrogen peroxide, Manganese chloride ($MnCl_2$*$4H_2O$), Proteinase K, Sodium citrate
**PAA:** DMEM, FCS, penicillin/streptomycin (PS), trypsin-EDTA.
**QIAGEN***:* Ni-NTA Superflow, QIAGEN Plasmid Maxi Kit, QIAquick Gel Extraction Kit, PCR purification kit
**Roche:** DNAse I, complete protease inhibitor cocktail, Shrimp Alkaline Phosphatase
**Roth:** Acetone, Ampicillin, 30% acrylamide mix, Boric acid, Bromophenol blue, Calcium chloride ($CaCl_2$), Dithiothreitol (DTT), EDTA, Glycerol, HEPES, Hydrogen chloride (HCl), Kanamycin, LB medium, LB agar, Magnesium sulphate ($MgSO_4$), Methanol, Paraformaldehyde (PFA), Potassium chloride (KCl) , Potassium phosphate ($KH_2PO_4$), Sodium hydroxide pellets, Sodium chloride (NaCl), Sodium phosphate ($Na_2HPO_4$*$7H_2O$ and $NaH_2PO_4$), TEMED, Triton X-100, Tryptone, Urea
**Serva***:* Bromphenol blue sodium salt
**Sigma***:* 2-mercaptoethanol, Aprotinin, Biotinylated SDS Molecular Weight Standard Mixture for SDS-PAGE (Molecular Weight Range 14,300 - 97,000 Da), BSA, Crystal violet, Cycloheximide, DMSO, EDTA, Ethidium bromide, Leupeptin, Luminol, Magnesium chloride, MG132, Nonidet P-40, Pepstatin, PIPES, Puromycin, Sodium bicarbonate, Sodium deoxycholate, Sodium dodecyl sulphate (SDS), Trypsin, Yeast extract
**Starlab**: Agarose

## 3.2. Solutions and buffers

**General**

| | |
|---|---|
| PBS | 137 mM NaCl<br>2,7 mM KCl<br>4,3 mM $Na_2HPO_4$*$7H_2O$<br>1,47 mM $KH_2PO_4$<br>pH 7,4 |
| TBS-T | 38 mM Tris<br>150 mM NaCl<br>0,1% Tween20<br>pH 7,6 |
| PBS-T | 1 ml Tween20 in 1 l PBS |

**Drosophila buffers**

| | |
|---|---|
| Squishing buffer | 10 mM TrisHCl pH 8,0<br>1 mM EDTA<br>25 mM NaCl<br>200 µg/µl Proteinase K |
| RIPA | 50 mM TrisCl pH 8,0<br>150 mM NaCl<br>0,1% SDS<br>0,5% sodium deoxycholate<br>1% Nonidet P-40<br>complete protease inhibitor cocktail |

**Protein biochemistry**

| | |
|---|---|
| Lysis buffer | 50 mM Tris-HCl (pH 7,4)<br>150 mM NaCl<br>1% Triton-X 100<br>complete protease inhibitor cocktail |

| | |
|---|---|
| Filter retardation 6x sample buffer | 7 ml TrisCl/SDS pH 6,8<br>3 ml glycerol<br>1,2 g SDS<br>0,46 g DTT |
| 6x SDS sample buffer | 7 ml 4x TrisCl/SDS pH 6,8<br>3 ml glycerol<br>1 g SDS<br>0,6 ml β-mercaptoethanol<br>10 mg bromophenol blue |
| 4x Tris-HCl/SDS, pH 6,8 | 0,5 M Tris<br>0,4% SDS |
| 4x Tris-HCl/SDS, pH 8,8 | 1,5 M Tris<br>0,4% SDS |
| 12% polyacrylamide resolving gel | 1,6 ml $H_2O$<br>2 ml 30% acrylamide mix<br>1,35 ml 4XTris-HCl/SDS pH 8,8<br>50 µl 10% ammonium persulfate<br>2 µl TEMED |
| 5% polyacrylamide stacking gel | 0,68 ml $H_2O$<br>170 µl 30% acrylamide mix<br>140 µl 4XTris-HCl/SDS pH 6,8<br>10 µl 10% ammonium persulfate<br>1 µl TEMED |
| Electrophoresis buffer | 10x solution:<br>250 mM Tris<br>1,9 M glycine<br>1% SDS |
| Transfer buffer | 25 mM Tris<br>192 mM glycine<br>20% methanol<br>pH 8,3 |
| ECL | solution 1:<br>100 µl 250 mM luminol<br>44 µl 90 mM p-coumaric acid |

1 ml 1 M Tris pH 8,5
8,85 ml $H_2O$

solution 2:
6 µl 30% $H_2O_2$
1 ml Tris pH 8,5
9 ml $H_2O$

**Calcium phosphate transfection**

HEPES buffer 2x
50 mM HEPES
250 mM NaCl
1,5 mM $Na_2HPO_4$
pH 6,9

**Nickel beads pull-down**

Buffer 1 (Lysis buffer)
6 M Guanidine-HCl
0.1 M $Na_2HPO_4/NaH_2PO_4$
0.01 M Tris-HCL
pH 8,0

Buffer 2
8 M Urea
0.1 M $Na_2HPO_4/NaH_2PO_4$
0.01 M Tris-HCL
pH 8,0

Buffer 3
8 M Urea
0.1 M $Na_2HPO_4/NaH_2PO_4$
0.01 M Tris-HCL
pH 6.3

**DNA buffers**

TBE buffer
37,2 g EDTA
540 g Tris
275 g boric acid
$H_2O$ up to 5 l

10x DNA loading buffer
5,7 ml glycerol
1 ml 1 M Tris, pH 8,0
1 ml 0,1 M EDTA
2,3 ml $H_2O$

**Competent cells**

| | |
|---|---|
| SOB medium | 0,5% yeast extract<br>2% tryptone<br>10 mM NaCl<br>2,5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$ |
| TB solution | 10 mM PIPES<br>15 mM $CaCl_2$<br>250 mM KCl<br>55 mM $MnCl_2$<br>pH 6,7 |

## 3.3. DNA constructs

The following cDNA constructs were used for transient and stable transfections:

| Construct | Vector | Restriction sites | Obtained from |
|---|---|---|---|
| Huntingtin Q15 1-139 (htt-wt) | pcDNA3 (Invitrogen) | XhoI, ApaI | W. Roth |
| Huntingtin Q117 1-139 (htt-mut) | pcDNA3 (Invitrogen) | XhoI, ApaI | W. Roth |
| Huntingtin Q15 1-139 eGFP | pcDNA3 (Invitrogen) | EcoRI, NotI | |
| Huntingtin Q117 1-139 eGFP | pcDNA3 (Invitrogen) | EcoRI, NotI | |
| Flag-BAG1 | pcDNA3 (Invitrogen) | BamHI, SalI | (Kermer et al., 2002) |
| Myc-BAG1ΔC (aa 1-190) | pcDNA3 (Invitrogen) | HindIII, XhoI | (Liman et al., 2005) |
| Siah1 | pcDNA3 (Invitrogen) | HindIII, XhoI | imaGenes (formerly RZPD) |
| HA-Siah1 ΔRING | pcDNA3 (Invitrogen) | HindIII, XhoI | |
| octa-His6-Ubiquitin | pMT 107 | NotI, EcoRI | F. Melchior |

## 3.4. Cloning

### 3.4.1. Primers

Primer design was performed using GENtle software.

Subcloning of flag-BAG1 to pcDNA3 vector:
Forward: 5' CGGTAGGATCCATGGACTACAAAGACGACAAGC 3'
Reverse: 5' CGGGTCGACTCATTCAGCCAGGGCCAA 3'
Restriction sites: BamHI/SalI

Subcloning of flag-BAG1 to pUAST fly expression vector
Forward: 5' CGTAGGCGGCCGCATGGACTACAAAGACG 3'
Reverse: 5' CGCTCGAGTCATTCAGCCAGGGC 3'
Restriction sites: NotI/XhoI

N-terminal HA tagging of Siah1
Forward: 5' GCAAGCTTATGTACCCATACGACGTCCCAGACTACG
CTAGCCGTC AGACTGCTACAGC 3'
Reverse: 5' CCTCTCGAGTCAACACATGGAAATAGTTACATTGATG
CCTAAATTGCCATTTTCTGCAAAAAGC 3'
Restriction sites: HindIII/XhoI

Deletion of the RING domain from Siah1
Forward: 5' GGCCCTTTGGGATCCATTCGCAACTTGGC 3'
Reverse: 5' CTCAAAAAGACTCGCCAAGTCATTGTTGG 3'

GAL4 (fly genotyping for elav-GAL4):
Forward: 5' AAGAGCATCCCTGGGCATAAA 3'
Reverse: 5' ATGAAGCTACTGTCTTCTATCG 3'

RKO (fly genotyping for cDNAs cloned into pUAST vector):
RKO 19: 5' AGAAGTAAGGTTCCTTCACAA 3'
RKO 20: 5' ACTGAAATCTGCCAAGAAGTA 3'

GAPDH primers for RT-PCR:
Forward: 5' CCCCACACACATGCACTTACC 3'
Reverse: 5' CCTACTCCCAGGGCTTTGATT 3'

Huntingtin primers for RT-PCR:
Forward: 5’ CGCAGAGTCAGATGTCAGGA 3’
Reverse: 5’ GAACTTCAGGGTCAGCTTGC 3’

### 3.4.2. PCR-amplification

Before amplification of the cDNA sequence of interest, the most suitable PCR conditions were established. Different annealing temperatures between 54 and 58°C, as well as varying denaturation, annealing and amplification times were used for the initial amplification. The conditions that resulted in the best yield of the PCR product were chosen for further amplification.

A PCR reaction mix typically contained:

5 µl of Pfu polymerase buffer (STRATAGENE)
200 nM of forward and reverse primers (Sigma)
200 µM of dATP, dCTP, dGTP, dTTP (Amersham)
20 ng of template DNA,
0,7 µl of PfuTurbo polymerase (STRATAGENE)
$H_2O$ to achieve a total volume of 50 µl.

The amplification was performed on a PCR machine from Peqlab (Cyclone 25).

A typical PCR reaction was:

| **Duration** | **Temperature** | **Cycles** |
|---|---|---|
| 60 sec | 94°C | 1 |
| 30 sec | 94°C | 30 |
| 30 sec | 55°C | |
| 45 sec | 72°C | |
| 10 min | 72°C | 1 |
| Hold | 4°C | |

The PCR product was purified using the PCR purification kit from Qiagen.

### 3.4.3. DNA restriction and purification

Restriction digest was performed using 2,5 µg of plasmid DNA and the whole amount of the PCR product. 0,5 µl of each endonuclease (New England Biolabs or Fermentas) was added and the reaction was performed in an appropriate buffer supplied by the producer at 37°C overnight. Following the digestion reaction, 1 µl of Shrimp Alkaline Phosphatase (Roche) was added to the sample containing the vector DNA for 1 hour at 37°C. This step is necessary to prevent recircularization of the plasmid. The enzymes were then heat-inactivated for 20 minutes at 65°C. Analysis of the DNA size was performed by agarose gel electrophoresis. To prepare the gel, 1% agarose was dissolved in TBE buffer in a microwave oven. Ethidium bromide solution was then added to the agarose solution (3 µl of 10 mg/ml stock solution to 50 ml of agarose solution) to enable visualization of the bands. DNA samples were mixed with 10x DNA loading buffer. The gel was run in 1x TBE buffer at 100 V. DNA bands were visualized by UV-light of 365 nm at Gel Documentation 2000™ UV-transilluminator (Bio-Rad) and the bands were excised using a scalpel. DNA extraction after gel electrophoresis was performed in accordance with QIAquick Gel Extraction Kit (QIAGEN) protocol. The DNA concentration in the final solution was measured at the Biophotometer (Eppendorf) at 260 nm.

### 3.4.4. DNA ligation and transformation in *E. coli*

For ligation, vector DNA and cDNA fragment were mixed in a molar ratio of 1:2 and brought to final volume of 17 µl with $H_2O$. The DNA was incubated for 5 minutes at 45°C to disrupt secondary structure formation. Following a brief cooling step on ice, 2 µl of ligation buffer and 1 µl of T4 ligase (New England Biolabs) were added. The ligation reaction was performed for 1 hour at room temperature. The ligase was then inactivated by heating the samples at 65°C for 20 minutes. 10 µl of the reaction was added to 100 µl of competent DH5α cells. The mixture was incubated on ice for 10 minutes and subsequently the bacteria were heat-shocked for 45 seconds at 42°C. Immediately after the heat-shock, 900 µl of LB medium was added and the cells were incubated for one hour with moderate shaking at 37°C and plated on LB agar plates containing ampicillin or kanamycin (100 µg/ml) for selection of clones. Bacterial clones were then picked and grown in 5 ml of LB medium with corresponding antibiotic at 37°C, 250 rpm shaking overnight. Plasmid DNA was purified using a miniprep kit. To verify whether the cloning was successful, the DNA was digested with the restriction enzymes used for cloning for 1 hour at 37°C and run on a 1% agarose gel. Clones containing an insert of the correct size were sent for sequencing to SEQLAB.

### 3.4.5. PCR mutagenesis

Siah1ΔRING construct was created using PCR mutagenesis. This method uses a proof-reading polymerase to read all the way around the plasmid. The primers were designed to border the deletion on two sides and were phosphorylated at the 5' end. The reaction mix contained:

5 µl 10x Pfu polymerase buffer
4 µl 10 mM dNTPs
0,2 µl of each primer (10 µM)
1 µl plasmid template (10 ng)
37,6 µl $H_2O$
2 µl Pfu polymerase

The PCR conditions were:

| Duration | Temperature | Cycles |
|---|---|---|
| 60 sec | 94°C | 1 |
| 30 sec | 94°C | 12 |
| 30 sec | 55°C | |
| 12 min | 68°C | |

The extension time was 2 minutes per kb of the plasmid. The reaction was cooled down to room temperature and 1 µl of DpnI restriction enzyme (New England Biolabs) was added for 1 hour at 37°C. DpnI is an enzyme which cuts only dam methylated DNA. The parental plasmid DNA is methylated in bacteria and is therefore cut to pieces while the nascent PCR DNA is left intact. 5 µl of the reaction was transformed into competent DH5α cells. Six colonies were minipreped, checked by restriction digest whether the insert is of the expected size and two of them were verified by sequencing (SEQLAB).

## 3.5. Preparation of competent cells for transformation

DH5α cells were cultured on an LB agar plate at 37°C overnight. 10 large colonies were picked from the plate and cultured in 250 ml SOB in a 1 L flask at 19°C with vigorous shaking to OD (600 nm)=0,5. The flask was placed on ice for 10 minutes. Cells were pelleted for 10 minutes at 3300 x *g*, 4°C. Cells were resuspended in 80 ml ice-cold TB solution, placed on ice for 10 minutes and spun again at 3300 x *g*

for 10 minutes (4°C). Cells were gently resuspended in 20 ml ice-cold TB solution and 1,4 ml DMSO. 300 µl aliquots were stored at -80°C.

### 3.6. RT-PCR

The enzymes and oligos were purchased from Invitrogen. RNA was isolated from cells transfected one day before using TRIzol according to manufacturer's protocol. 1 µg of total RNA was digested with DnaseI for 1 hour in 37°C (final volume 10 µl, 2 mM $MgCl_2$). DnaseI was then inactivated by incubation at 70°C for 10 minutes. Reverse transcription was performed as described by the manufacturer using Superscript III RT enzyme in the presence of 0,5 µl of oligo(dT)s and 0,5 µl of random hexamers. The reaction was terminated at 70°C for 10 minutes. To remove RNA, 1µl of RnaseH was added for 20 minutes at 37°C. 10% of the reverse transcription reaction was then used for the PCR using primers specific for htt or GAPDH with the following cycling conditions:

| Duration | Temperature | Cycles |
|---|---|---|
| 2 min | 94°C | 1 |
| 30 sec | 94°C | 17 |
| 30 sec | 55°C | |
| 45 sec | 72°C | |

5% of the PCR reaction was subjected to agarose gel electrophoresis.

### 3.7. Cell culture

CSM14.1 wt, $STHdh^{Q111}$ and $STHdh^{+}$ cells (a kind gift from E. Cattaneo) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) , 100 U/ml penicillin and 100 µg/ml streptomycin at 32°C, 5% $CO_2$ supply. Stably transfected CSM-BAG1 (Kermer et al., 2002) and CSM-BAGΔC (Liman et al., 2005) cells were maintained in the same medium with an addition of puromycin (8 µg/ml). HEK293T cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin in 37°C, 5% $CO_2$. After reaching approximately 70% confluency, cells were split 1:10. The medium was removed, cells were washed with 2 ml of PBS and 2 ml of 0.05% trypsin/ 0.02% EDTA solution was added to the culture dish and left

in the incubator at 37°C for 5 (HEK293T, STHdh$^{Q111}$ and STHdh$^{+}$ cells) or 15 (CSM 14.1 cells) minutes until the cells started to detach from the dish. Trypsin activity was stopped by addition of 2 ml of the cell culture medium to the dish. The mix containing detached cells was transferred to a 15 ml Falcon tube and spun down at 390 x *g*, 4°C, 5 minutes. After removing the supernatant, the cell pellet was resuspended in 1 ml of the culture medium and seeded on culture dishes.

## 3.8. Stable transfections

50-70% confluent STHdh$^{Q111}$ and STHdh$^{+}$ cells were transfected with flag-BAG1 plasmid containing puromycin resistance cassette or a mock empty vector using Lipofectamine 2000 reagent according to manufacturer's protocol. Selection with 8 µg/ml puromycin was started 24 hours after transfection. After 5 days, the cells were trypsinized, counted and seeded in 96-well plates (0,5 cell per well) with selection medium. 3 to 4 weeks later, wells containing single clones were identified by light microscopy. The cells were then transferred to larger plates for expansion and further processing. Expression of flag-BAG1 was confirmed by western blotting.

## 3.9. Transient transfections

Most transfections were done using Lipofectamine 2000 reagent. In case when large amount of cells needed to be transfected, a more cost-efficient calcium phosphate method was used.

### 3.9.1. Lipofectamine 2000

To transfect cells seeded on a 6 well plate, 800-1600 ng of DNA was diluted in 100 µl of OptiMEM medium. 4 µl of Lipofectamine 2000 was diluted in 100 µl of OptiMEM. After 5 minutes incubation at room temperature, the DNA solution was mixed with the Lipofectamine 2000 solution and incubated for another 20 minutes. In the meantime, cell culture medium was exchanged for a fresh one without antibiotics to avoid cell death. DNA•Lipofectamine complexes were then added dropwise to the cells. Gene expression was assayed 24 to 48 hours later. For transfections in dishes of different size, the amount of reagents was scaled up or down according to the relative surface area of the dish.

### 3.9.2. Calcium phosphate transfections

To transfect cells plated on a 10 cm dish, 45 µg DNA was mixed with 125 µl of 2 M $CaCl_2$ and $H_2O$ to achieve a total volume of 1 ml. 1 ml of 2x HEPES buffer was added, mixed and incubated for 15 minutes at room temperature. During the incubation time, fresh medium was added to the cells. Following the incubation time, the mixture was added dropwise to the cells. Twelve hours post transfection cell were washed in PBS and fresh medium was added. Gene expression was assayed 36 hours later.

## 3.10. Cell death experiments

$STHdh^{Q111}$ and $STHdh^{+}$ cells stably transfected with flag-BAG1 or empty vector were plated on 96 well plates at 8000 cells/well (8 wells per condition) in permissive temperature (32°C). 24 hours later the cells were transferred to non-permissive temperature (39°C). Cell death was evaluated for seven days by crystal violet staining. Growth medium was removed from cells and 50 µl of crystal violet solution (0,5% in 20% methanol, v/v) was added for 10 minutes. The solution was then washed out with water and plates were left to dry on air for 24 hours. Subsequently, 100 µl of sodium citrate solution (0,1 M in 50% ethanol) was added to the wells to dissolve dried out crystal violet, and the staining intensity was measured using an ELISA plate reader (rainbow, TECAN) at 550 nm.

## 3.11. Protein extracts preparation

Cells were washed and scraped in ice-cold PBS and pelleted in Eppendorf tubes at 300 x *g*, 4°C, using a standard table-top centrifuge (Heraeus). Cells were lysed for 15 minutes at 4°C, followed by 10 minutes centrifugation at 16000 x *g,* at 4°C. The pellets, which were further used for the filter retardation assay, were resuspended in the lysis buffer and sonicated for 30 seconds at 40% power, at 4°C (SONOPULS, Bandelin electronics). Protein concentration was measured using the BCA Protein Assay Kit (Pierce).
Subcellular fractionation was performed using ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem).

## 3.12. Filter retardation assay

For the filter retardation assay (Wanker et al., 1999), protein extracts were diluted to 90 µl with lysis buffer and 15 µl 6x sample buffer was added. Samples were heated at 98°C for 3 minutes and filtered through a 0.2-µm cellulose

acetate membrane (Schleicher & Schuell, Dassel, Germany) using a dot-blot filtration unit (Schleicher & Schuell, Dassel, Germany). The membranes were washed briefly with TBS-T and further processed for immunodetection.

### 3.13. Co-immunoprecipitation

Cell lysates were prepared as described above. Flag-affinity beads (Sigma) were washed twice with lysis buffer and 25 µl beads were added to each tube. The beads were then incubated with cellular lysates for 2 hours at 4°C with rotation. Subsequently, they were spun down, washed twice with the lysis buffer and twice with TBS buffer (400 µl, 5 minutes each washing step). Proteins bound to the beads were eluted by adding 20 µl 2X SDS sample buffer for 5 minutes at 95°C.

### 3.14. Nickel beads pull-down

Two 10 cm dishes of HEK293T cells per condition were transfected using calcium phosphate transfection (45 µg DNA per plate, 15 µg per construct per plate). To block proteasomal degradation of ubiquitinated proteins, cells were incubated with 10 µM MG132 for 12 hours. Cells from one 10 cm plate were lysed in 3 ml buffer 1 supplemented with 10 mM NEM, 10 mM Imidazole, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin, followed by 30 seconds sonication (30%, constant mode, SONOPULS, Bandelin electronics). 200 µl of the lysate was methanol/chloroform precipitated to later asses input protein levels. Lysates were incubated with 200 µl Ni-NTA resin (Qiagen), previously washed with buffer 1, for 2,5 hours at 4°C.

Ni-beads were extensively washed with 10 ml of the following buffers:

1. Buffer 1, supplemented with 10 mM NEM, 10 mM Imidazole, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin
2. Buffer 2, supplemented with 10 mM NEM, 10 mM Imidazole, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin
3. Buffer 3 pH 6,3, supplemented with 10 mM NEM, 20 mM Imidazole, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin, 0.02% TritonX
4. Buffer 3 pH 6,3, supplemented with 10 mM NEM, 10 mM Imidazole, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin, 0.02% TritonX
5. Buffer 3 pH 6,3, supplemented with 10 mM NEM, 10 mM Imidazole, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin, 0.01% TritonX
6. Buffer 3 pH 6,3, supplemented with 20 mM NEM, 10 mM Imidazole, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin,

Bound proteins were eluted with 250 µl Buffer 3 supplemented with 250 mM

Imidazole. After methanol/chloroform precipitation, pellets were resuspended in 35 µl SDS sample buffer.

### 3.15. Methanol/chloroform precipitation

200 µl of the protein extract was mixed with 600 µl methanol and 200 µl chloroform. 600 µl $H_2O$ was added and the samples were centrifuged for 5 minutes at 9500 x *g* in a table top centrifuge. The upper layer containing $H_2O$ and methanol was discarded. 600 µl methanol was added, mixed and the samples were centrifuged again for 5 minutes at 9500 x *g*. The supernatant was discarded and the precipitate was let to dry on air for 15 minutes. The pellet was resuspended in 50 µl 1X SDS sample buffer and sonicated to facilitate dissolving.

### 3.16. SDS-polyacrylamide gel electrophoresis (SDS- PAGE)

Two-phase gels (12% resolving gel and 5% stacking gel) were used for separation of the proteins according to their molecular weight. To define the molecular weight of loaded proteins, molecular weight marker was loaded and separated in parallel. Equal volumes of protein samples diluted to the same concentration and 6x SDS sample buffer were mixed, heated at 95°C for 5 minutes and loaded in the gel wells. SDS-PAGE was run at 4°C in electrophoresis buffer. The equipment used was Mini-PROTEAN electrophoresis system from Bio-Rad. Electric field of 70 V was applied for 15 minutes to allow samples to enter the gel and then increased to 100 V and kept constant until the desired separation of proteins (as judged by the prestained molecular weight marker) was achieved.

### 3.17. Immunoblotting

For western blotting, a Mini Trans-Blot Cell setup (7.5 x 10 cm blotting area, Bio-Rad) was used. Following SDS-PAGE, the polyacrylamide gel was placed between two sheets of blotting pads, Whatman filter paper and a nitrocellulose membrane (Applichem), all equilibrated in transfer buffer. The “sandwich” was placed in the Mini Trans-Blot Cell. Amperage of 400mA was applied for 90 minutes at 4°C. After the blotting step, the membrane was incubated in blocking solution (5% milk in TBS-T) for 1 hour at room temperature to avoid unspecific binding of the antibody. Incubation with primary antibodies was carried out at 4°C overnight and with secondary HRP-conjugated antibodies for 1h at room

temperature. The membrane was washed 3 x 20 minutes with TBS-T after each antibody incubation. To develop membranes, equal volumes of ECL-1 and ECL-2 reagents were mixed and applied for 2 minutes onto the membrane. Films (Amersham, Hyperfilm ECL) were exposed to the membrane for various amounts of time in order to achieve the desired signal intensity and developed using AGFA Curix 60 table-top processor.

### 3.18. Densitometry analysis

To quantify Western blot protein bands, ImageJ software was used. The blot images were opened in the program as tiff files. A same size rectangular selection was drawn around each band and lanes were selected using Analyze>Gels>Select Lane function. Histograms representing the intensity of pixels in the selected areas were created using Analyze>Gels>Plot Lanes function. The area under the histograms, representing pixel intensity in the selected area, was limited using the straight line selection in order to integrate the signal. The values of the histogram area were obtained by clicking inside the histogram with the wand tracing tool. Values corresponding to the protein of interest were normalized by dividing them by the values of the loading control (actin, β-tubulin or histone H2B) to account for differences in the amount of protein loaded.

### 3.19. Immunocytochemistry

Cells cultured on cover-slips were washed in ice-cold PBS and fixed in cold PBS containing 4% paraformaldehyde, pH 7,4 for 10 minutes at room temperature, followed by several washing steps in PBS. Permeabilization was performed with -20°C acetone for 10 minutes with subsequent preblocking in PBS containing 3% bovine serum albumin and 0,1% Triton-X 100. Cells were incubated in blocking solution containing 1C2 antibody (Chemicon, dilution 1:1000) or BAG1680 antibody (1:1000) for 1 hour at room temperature. Cells were washed three times in PBS-T and incubated with Cy5-conjugated secondary anti-mouse antibody (Jackson ImmunoResearch Laboratories, dilution 1:400 in blocking solution) or Cy3-conjugated secondary anti-rabbit antibody (Jackson ImmunoResearch Laboratories, dilution 1:400 in blocking solution) for 1 hour at room temperature. Cells were again washed three times in PBS-T and mounted on microscope slides using moviol (Calbiochem). Before the last washing step, DAPI solution was applied to cells for 2 minutes to stain the nuclei. Samples were analyzed by epifluorescence (Axioplan, Zeiss) or confocal (TCS SP2 AOBS, Leica) microscopy.

## 3.20. Antibodies

| Antibody | Raised in | Clonality | Dilution | Obtained from |
|---|---|---|---|---|
| CAG53B (raised against polyQ stretches) | rabbit | polyclonal | 1:2000 | E.E. Wanker (Davies et al., 1997) |
| HD1 (recognizes both htt-wt and htt-mut) | rabbit | polyclonal | 1:2000 | E.E. Wanker (Scherzinger et al., 1997) |
| 1C2 (raised against polyQ stretches) | mouse | monoclonal | 1:1000 | Chemicon |
| BAG1680 | rabbit | polyclonal | 1:1000 | J.C. Reed (Kermer et al., 2002) |
| Siah1 | goat | polyclonal | 1 :1000 | Everest Biotech |
| βtubulin | mouse | monoclonal | 1 :1000 | Sigma |
| Histone H2B | rabbit | polyclonal | 1 :2000 | Abcam |
| actin | mouse | monoclonal | 1 :5000 | Chemicon |
| HA.11 | mouse | monoclonal | 1 :1000 | Covance |
| Proteasome α7 subunit | mouse | monoclonal | 1 :1000 | BIOTREND Chemikalien |
| HRP- conjugated anti-mouse | goat | polyclonal | 1 :2000 | Santa Cruz |
| HRP-conjugated anti-rabbit | goat | polyclonal | 1 :4000 | Santa Cruz |
| HRP-conjugated anti-goat | donkey | polyclonal | 1 :2000 | Santa Cruz |

## 3.21. *D. melanogaster* techniques

### 3.21.1. *Drosophila* Strains

Fly strains were maintained under standard laboratory conditions.

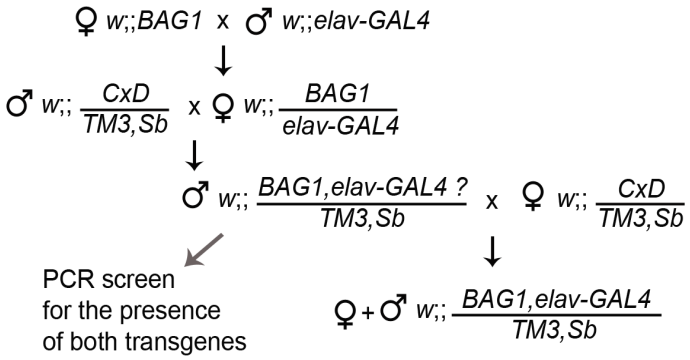
| Fly strain | Obtained from |
|---|---|
| y[1] w[*]; CxD/TM3, Sb[1] Ser[1] | Bloomington |
| elav-GAL4 | Bloomington |
| UAS-eGFP | A. Voigt (unpublished) |
| w; P(w+mC =UAS-Q93httexon1)4F1 | J.L. Marsh (Steffan et al., 2001) |

### 3.21.2. Generation of UAS-BAG1 flies

cDNA coding for mouse flag-BAG1 was cloned into the pUAST *Drosophila* expression vector using NotI and XhoI restriction enzymes. Transgenic *Drosophila* lines were generated by microinjection following standard procedures. Several lines of BAG1 flies with the transgene incorporated on the second or third chromosome were obtained. The w;;UAS-flagBAG1 #8.2 line was used for further experiments.

### 3.21.3. Recombination of BAG1 flies with elav-GAL4 flies

To recombine BAG1 gene with the pan-neuronal driver elav-GAL4, the following crossing scheme was set up:




### 3.21.4. Fly genotyping

Flies were placed in 200 µl eppendorf tubes, squished with a pipette tip, and 50 µl of squishing buffer was added. Flies were incubated for one hour at room temperature. Proteinase K was then heat inactivated at 94°C for 4 minutes. The following PCR was set up:

5 µl 10x high yield buffer (Peqlab)
1 µl of forward and reverse primer (10 µM)
1 µl of dNTPs (10 mM)
1 µl of the fly extract
1 µl of Taq polymerase
40 µl $H_2O$

The cycling conditions were:

| Duration | Temperature | Cycles |
|---|---|---|
| 2 min | 94°C | 1 |
| 30 sec | 94°C | 30 |
| 30 sec | 55°C | |
| 45 sec | 72°C | |
| 5 min | 72°C | 1 |
| Hold | 4°C | |

### 3.21.5. Preparation of protein extracts from fly heads

10 flies were placed in an eppendorf tube, flash frozen in liquid nitrogen and vortexed at full speed for 15 seconds. Freezing and subsequent vortexing makes the fly head separate from the body. 20-25 fly heads were then put in a tube containing homogenization beads and 100 µl RIPA buffer and homogenized using Precellys 24 homogenizer (Peqlab) for 30 seconds at 6500 rpm. Fly extracts were centrifuged for 15 minutes at 16000 x *g* in a table top centrifuge and transferred to a clean tube. Protein extracts were sonicated for 30 seconds at 40% power (SONOPULS, Bandelin electronics) and again centrifuged for 15 minutes at 16000 x *g* to get rid of all the chitinous structures. Protein extracts were then subjected to SDS-PAGE and immunoblotting.

### 3.21.6. Photoreceptor cell loss analysis

Two imaging methods were used to visualize rhabdomeres of the compound eye of the fly. In the first method whole flies were laterally attached to a plastic dish using transparent nail polish and imaged using a 20x water immersion objective (Zeiss) and DAPI filter settings at an Axioplan Zeiss microscope (Pichaud and Desplan, 2001; Stark and Thomas, 2004). In the second approach flies were decapitated and heads were attached to a microscope slide with nail polish (Jackson et al., 1998). The photoreceptors were visualized with a 63x oil immersion objective, white light using the same microscope.

### 3.22. Statistical analysis

All experiments that involved counting for statistical analysis were performed in a blind manner. All the values are presented as mean ± standard error of the mean (SEM). For each time point and experimental condition n≥3 was used. Pairwise comparisons of values were performed by paired two-tailed Student's *t*-test. Comparisons of three or more samples were performed by one way ANOVA test.

# 4. RESULTS

## 4.1. BAG1 modulates subcellular localization of mutant huntingtin

We overexpressed N-terminal fragments of wild type (Q15) or mutant (Q117) huntingtin (htt-wt or htt-mut) in immortalized rat CSM 14.1 neuronal cells. Subcellular fractionation and immunoblotting revealed that htt-wt is localized predominantly in the cytosolic fraction (Fig. 4.1a) and gives almost no signal in the nuclear fraction, which is in line with previous reports showing that it is mainly distributed diffusely within the cytosol (DiFiglia et al., 1995; Gutekunst et al., 1995; Trottier et al., 1995). Mutant huntingtin, on the other hand, was found both in the cytosol and in the nucleus. This is also in accordance with previous studies demonstrating that nuclear accumulation leading to toxicity occurs in case of the mutant protein (Saudou et al., 1998; Peters et al., 1999). In cells stably expressing BAG1, however, nuclear htt-mut levels were decreased by about 60% when compared to wild type cells (Fig. 4.1b), an effect which could also be visualized by immunocytochemistry using an antibody directed against the polyglutamine stretch (Fig. 4.1c). In wild type cells there is a clear accumulation of htt-mut in the nucleus (Fig. 4.1c, left panel), whereas in BAG1 expressing cells the nuclear staining is markedly weaker (Fig. 4.1c, right panel). In comparison, BAGΔC, which is a deletion mutant of BAG1 lacking the BAG domain and therefore unable to bind Hsp70, does not diminish the amount of htt-mut in the nucleus (Fig. 4.1d).

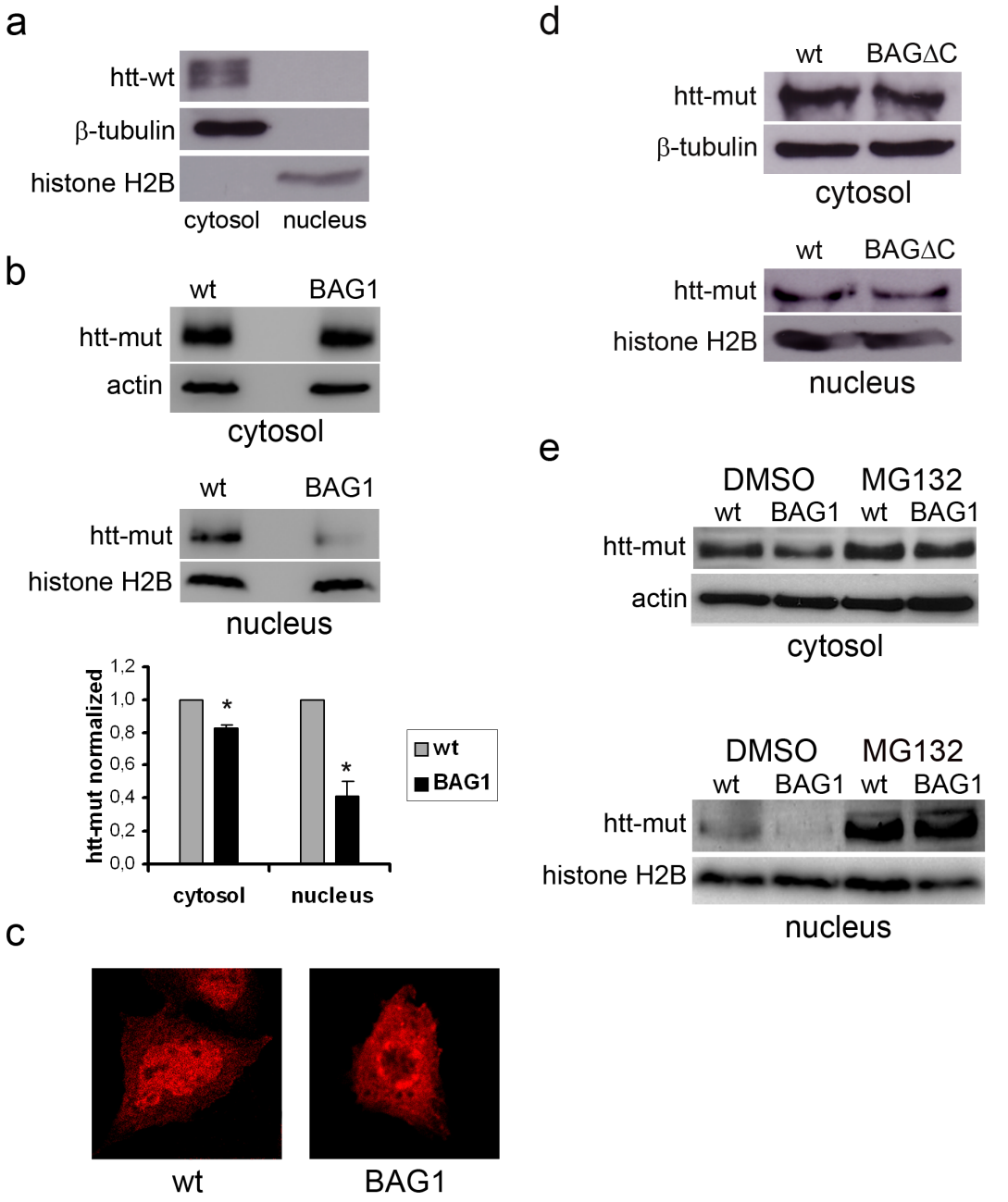



Fig. 4.1 BAG1 influences htt-mut subcellular localization.
CSM 14.1 cells were transfected with htt-wt or htt-mut and subjected to subcellular fractionation and immunoblotting. Htt-wt is found mainly in the cytosolic fraction (a), while htt-mut is localized both in the cytosol and in the nucleus (b). Cells stably expressing BAG1 have significantly less htt-mut in the nuclear fraction (b). The bottom graph is a densitometric quantification of three independent experiments (b, * $p<0.05$, Student's *t*-test). Confocal images of wild type and BAG1 stable cells transfected with htt-mut and stained with an antibody directed against the polyQ stretch (c). BAGΔC does not prevent nuclear accumulation of htt-mut (d). Inhibition of the proteasome by MG132 (10 μM for 12 hours) abolishes the effect BAG1 has on nuclear levels of htt-mut (e).

The CSM 14.1 cells which we used for the experiments were stably transfected with BAG1 and BAGΔC constructs, meaning they were derived from a single cell during antibiotic selection process (Kermer et al., 2002). To exclude artefacts caused by possibly unequal expression levels in different clonal cell lines, we examined htt-mut protein content by western blotting after transient transfection in wild type, stable BAG1 and stable BAGΔC cells without documenting significant differences (Fig. 4.2a). Moreover, we compared expression levels of htt-mut on mRNA level by RT-PCR in all three cell lines and did not detect any variation (Fig. 4.2b). We also obtained identical result of htt-mut subcellular localization in a transient transfection of the nonneuronal HEK293T cell line (Fig. 4.3a), indicating that the effect is specifically due to BAG1 overexpression.

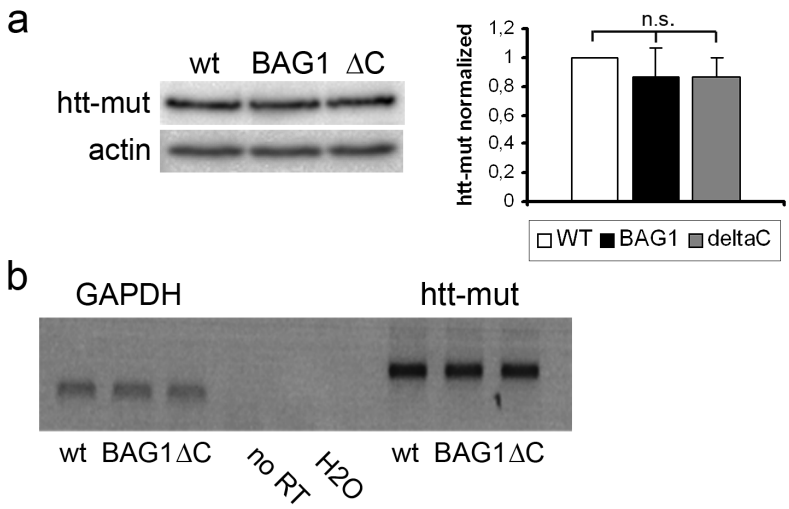



Fig. 4.2 Expression levels in CSM 14.1 wt, BAG1 and BAGΔC (ΔC) cells are comparable. Expression levels of htt-mut protein after a transient transfection are the same among the cell lines. The graph is a densitometric quantification of three independent experiments (ANOVA test, not significant) (a). mRNA levels of transiently transfected htt-mut are similar in all three cell lines as seen by RT-PCR (b).

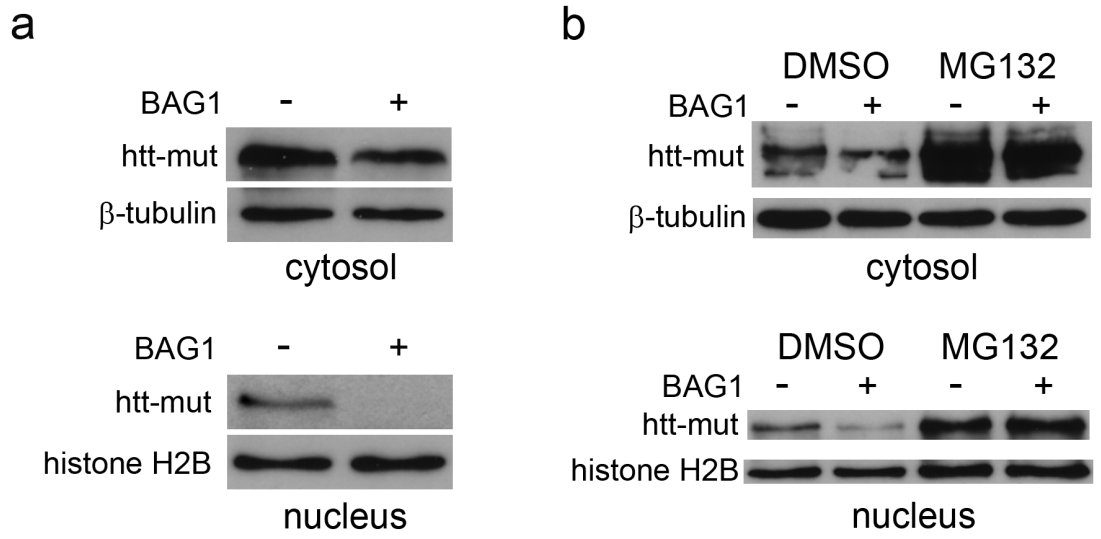



Fig. 4.3 BAG1 influences htt-mut subcellular localization in HEK293T cells.
HEK293T cells were transfected with htt-mut and BAG1 or a mock empty vector and subjected to subcellular fractionation and immunoblotting. BAG1 decreases the amount of htt-mut in the nucleus (a). Inhibition of the proteasome by MG132 (5 μM for 12 hours) abolishes the effect BAG1 has on nuclear levels of htt-mut (b).

There could be several explanations for the fact that BAG1 reduces the amount of htt-mut in the nucleus. Either BAG1 influences htt-mut shuttling between the cytoplasm and the nucleus, affecting nuclear import or export, or it stimulates its degradation. To test the degradation hypothesis, we blocked the proteasome by application of an inhibitor MG132 and saw that it abolishes the effect of BAG1 on nuclear levels of htt-mut (Fig. 4.1e and Fig. 4.3b), suggesting that BAG1 might indeed influence htt-mut removal by the UPS.

With regard to nucleo-cytoplasmic shuttling of huntingtin, not much is known about its mechanism. A recently published report (Bae et al., 2006) showed that mutant huntingtin fragment forms a complex with GAPDH and Siah1 prior to its nuclear import. Since BAG1 is known to bind to and inhibit Siah1 (Matsuzawa et al., 1998), it was a plausible hypothesis that BAG1 might affect htt-mut subcellular localization through inhibition of Siah1. In this regard, we detected reduced levels of endogenous Siah1 in CSM 14.1 cells stably expressing BAG1 (Fig. 4.4a). The same holds true when we co-expressed a

mutant of Siah1 lacking its RING domain (Siah1-ΔRING) together with BAG1 in HEK293T cells (Fig. 4.4a). BAG1 decreases the levels of endogenous Siah1 in this cell line as well as the amount of exogenous Siah1-ΔRING. We chose to use a deletion mutant of Siah1 because the full length protein is turned-over very rapidly and we could not obtain sufficient levels of expression necessary for our experiments. Siah1 is a RING-type E3 ligase for ubiquitin, and through its RING domain it is able to mediate auto-ubiquitination which leads to its degradation by the proteasome (Hara et al., 2005). As Siah1 is a short-lived protein and readily degraded by the proteasome, the effect of proteasomal inhibition on htt-mut nuclear levels described above (Fig 4.1e and Fig. 4.3b) could not only be explained by a direct effect on mutant huntingtin degradation but also be an indirect effect of accumulation of Siah1, facilitating nuclear import

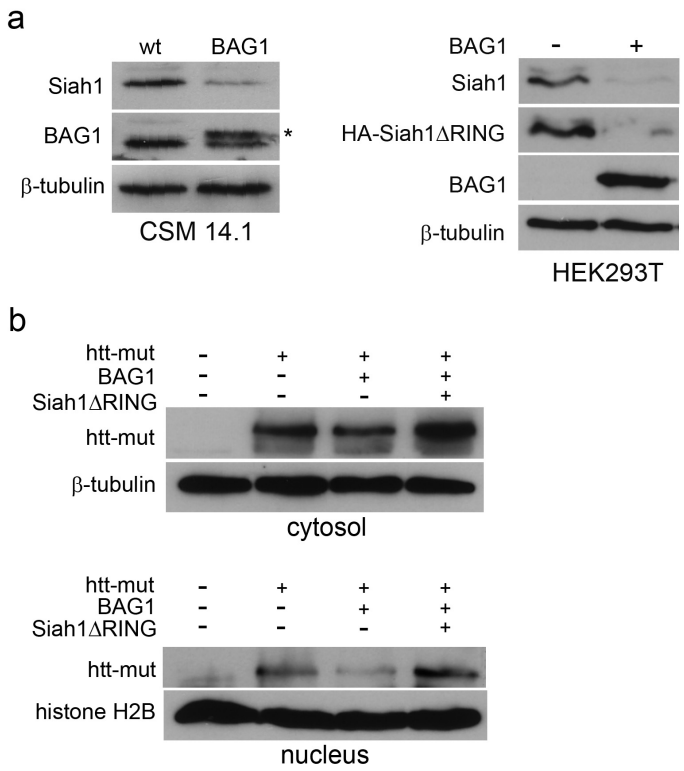



Fig. 4.4 Siah1 counteracts the effect of BAG1 on htt-mut subcellular localization.
CSM 14.1 cells stably expressing BAG1 have lower levels of Siah1 (a, left panel). The asterisk indicates the stably transfected flag-BAG1, which migrates slower than the endogenous BAG1. BAG1 reduces the amount of both endogenous Siah1 and overexpressed Siah1ΔRING mutant in HEK293T cells (a, right panel). Expression of Siah1ΔRING in HEK293T cells leads to accumulation of htt-mut in the nucleus even in the presence of BAG1 (b).

of mutant huntingtin (Fig. 4.5). To test this hypothesis, we performed subcellular fractionation experiments co-transfecting mutant huntingtin together with BAG1 and Siah1-ΔRING in HEK293T cells. We indeed observed that overexpression of Siah1-ΔRING can overcome the effect of BAG1 with more mutant huntingtin accumulating in the nucleus (Fig. 4.4b).

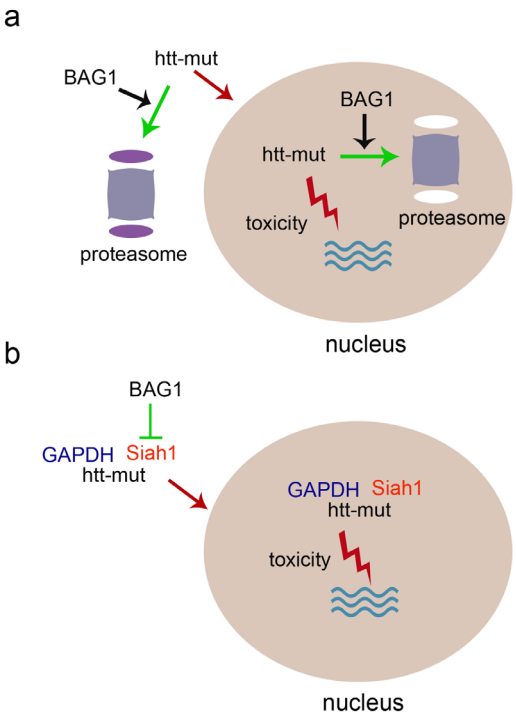



Fig. 4.5 Two models of how BAG1 might reduce the amount of htt-mut in the nucleus.
(a) BAG1 promotes htt-mut degradation through the proteasome.
(b) Htt-mut forms a complex with GAPDH and Siah1 in order to enter the nucleus. BAG1 binds and inhibits Siah1, preventing htt-mut from entering the nucleus.

## 4.2. Reduction of mutant huntingtin aggregation by BAG1

Since various chaperones have been shown to modulate the aggregation process of mutant huntingtin, we investigated whether BAG1 as a co-chaperone also exerts such properties. First, we expressed mutant huntingtin fused to a C-terminal eGFP and observed that it forms multiple aggregates in the cytoplasm, the perinuclear region and the nucleus, as described before (Fig 4.6a) (DiFiglia et al., 1997; Becher et al., 1998). Over time, multiple aggregates would coalesce forming one big aggresome-like inclusion localized in the perinuclear region (Fig. 4.6c) (Johnston et al., 1998; Taylor et al., 2003). Co-staining of the cells with a BAG1 antibody revealed that BAG1 is enriched in some of huntingtin aggregates (Fig. 4.6b left panel) or forms a circle around them (Fig 4.6b right panel), suggesting that it may be involved in the cellular response to mutant huntingtin aggregation (Sherman and Goldberg, 2001). These results add BAG1 as another chaperone to the list of those that are recruited to inclusion bodies formed by polyQ expansion proteins (see “Chaperone mediated neuroprotection”).

We then quantified htt-mut aggregation by counting how many, out of all transfected cells, contain inclusion bodies at different times post-transfection. We observed that CSM 14.1 cells stably expressing BAG1 develop significantly less inclusion bodies at all time points investigated (Fig. 4.7a). In contrast, cells stably expressing BAGΔC mutant did not display reduced inclusion body formation. We confirmed this *in situ* observation employing a filter retardation assay (Wanker et al., 1999). This method takes advantage of the biochemical properties of huntingtin aggregates that are detergent-insoluble. Briefly, the insoluble part of the protein lysate containing the aggregates and remaining after the centrifugation step (pellet) is resuspended in the lysis buffer, sonicated and boiled in the presence of 2% SDS. This denatures all soluble proteins, whereas the aggregates are resistant to the treatment. The samples are then filtered through a cellulose acetate membrane with 0,2 µm pore size.

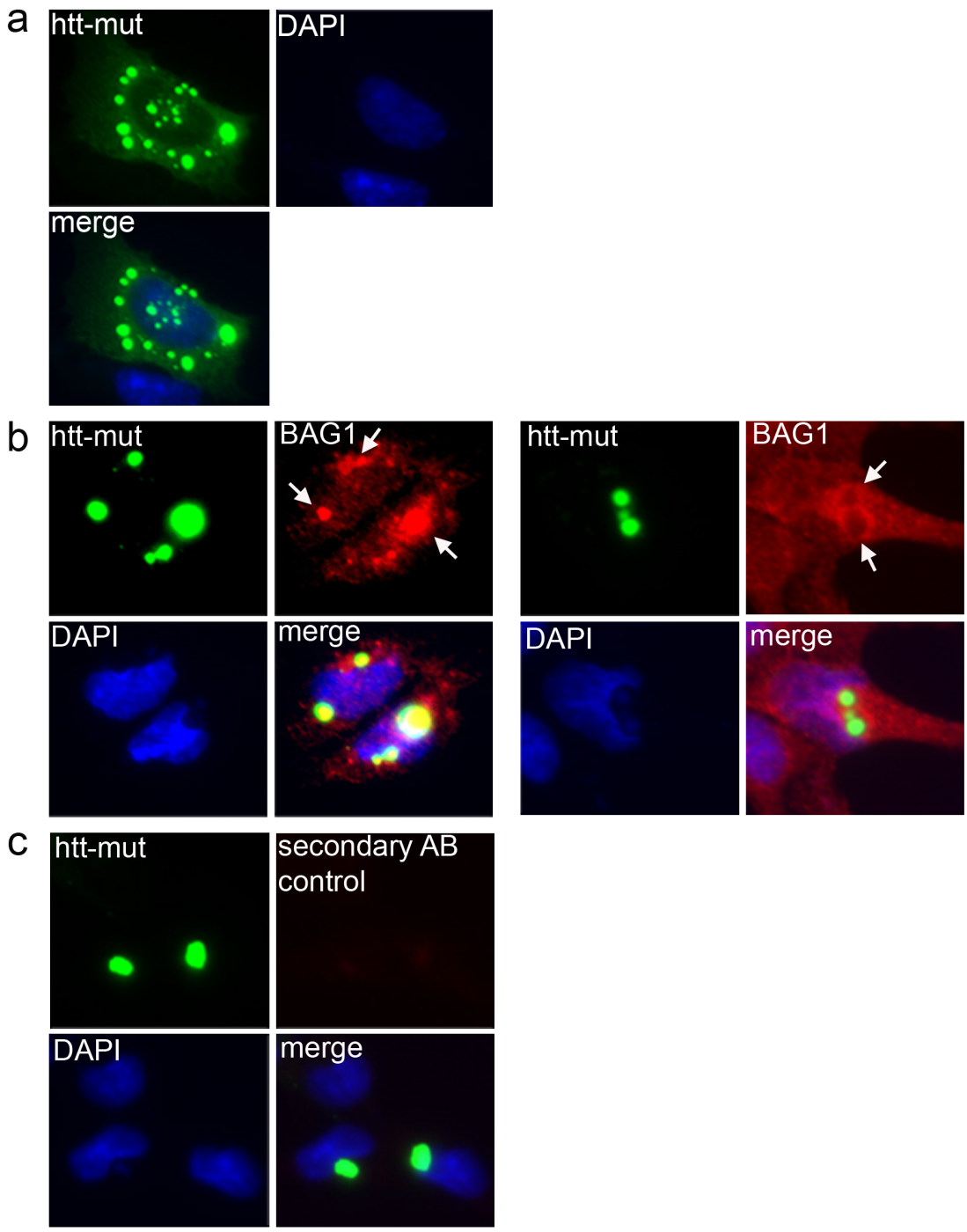



Fig. 4.6 BAG1 is recruited to htt-mut inclusion bodies.
CSM 14.1 cells were transfected with htt-mut fused to eGFP and counterstained with an antibody directed against BAG1. Htt-mut_eGFP forms multiple inclusions in the cell body, the perinuclear region and the nucleus (a), which eventually coalesce to form a single inclusion body usually localized perinucleary (c). BAG1 colocalizes with htt-mut in inclusion bodies (b, left panel, arrows). Sometimes BAG1 staining was seen not directly in the inclusion but in a ring shape surrounding it (b, right panel, arrows).

All denatured soluble proteins pass through the pores of the membrane, while big aggregates are stuck onto it and can be later visualized by antibody staining. Employing this assay, we again saw that BAG1, but not BAGΔC, decreases the amount of htt-mut aggregation (Fig. 4.7a).

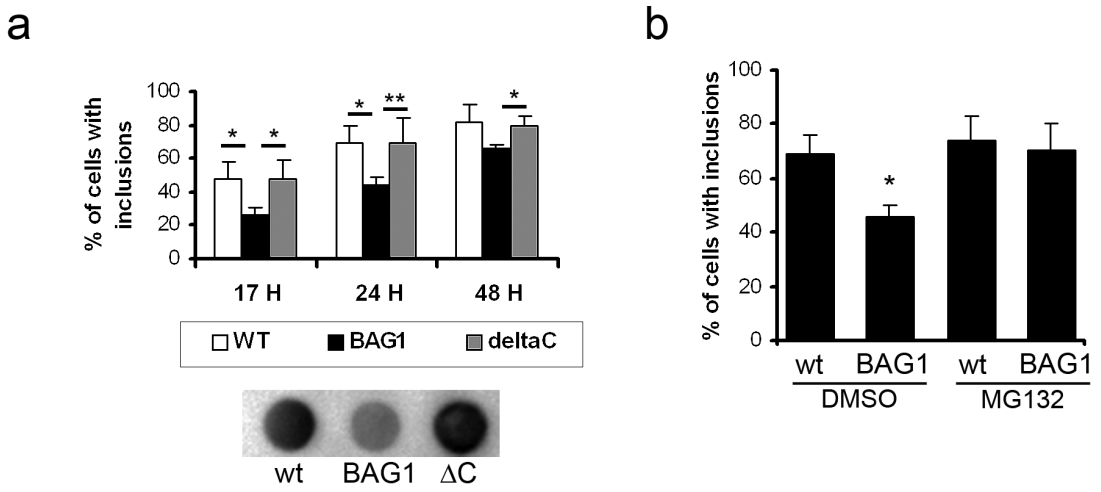



Fig. 4.7 BAG1 decreases htt-mut aggregation in an Hsp70- and proteasome-dependent manner.

CSM 14.1 wt, BAG1 and BAGΔC (ΔC) cells were transfected with htt-mut fused to eGFP. The percentage of cells that contained inclusions was obtained by counting. Cells stably expressing BAG1 are less likely to develop inclusions than wild type cells or cells stably expressing BAGΔC mutant at all time points post-transfection (a, top). Reduction in aggregate formation can also be observed in a filter retardation assay (a, bottom), where captured aggregates were detected with an antibody directed against the polyQ stretch. Blocking the proteasome with MG132 (10 μM, 17 hours) abolishes the effect of BAG1 on htt-mut aggregation (b). At least 400 cells were scored for each condition. The data represent mean values and SEM of three independent experiments (Student's *t*-test, *, $p<0.05$, **, $p<0.01$, a; ANOVA,*, $p<0.05$, b).

## 4.3. Increased turn-over of mutant huntingtin in the presence of BAG1

As was the case for subcellular localization of mutant huntingtin shown before, inhibition of the proteasome by MG132 abolished the effect of BAG1 on huntingtin aggregation (Fig 4.7b), pointing again to the possibility that BAG1 facilitates the degradation of mutant huntingtin. Furthermore, by co-immunoprecipitation, we were able to show that BAG1 interacts with the α7 subunit of the proteasomal core (Fig. 4.8), what confirms a report that BAG1 associates with the proteasome through its ubiquitin-like domain (Luders et al., 2000).

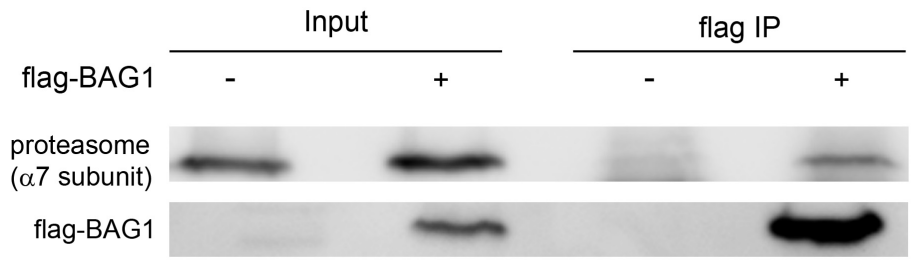



Fig. 4.8 BAG1 interacts with the α7 subunit of the proteasomal core particle.
HEK293T cells were transfected with flag-BAG1 or a mock empty vector. Cell lysates were subjected to co-immunoprecipitation using flag-affinity beads (flag IP), followed by immunoblotting. The membranes were probed with antibodies against BAG1 or the α7 proteasomal subunit. First two lanes show the input from cells transfected with an empty vector control of flag-BAG1. The α7 proteasomal subunit is pulled down by flag-BAG1. No α7 is detected after immunoprecipitation in the negative control.

To substantiate our results obtained with the proteasomal inhibitor MG132 and to investigate whether BAG1 can indeed directly influence htt-mut degradation, we performed a cycloheximide chase experiment. Cycloheximide is a bacterial inhibitor of eukaryotic protein translation frequently used to determine the half-life of proteins *in vitro*. We observed that htt-mut is indeed cleared faster in cells stably expressing BAG1 with a half-life of ~3,3 hours, as compared to wild type cells in which it had a half-life of ~4,9 hours (Fig. 4.9a, b).

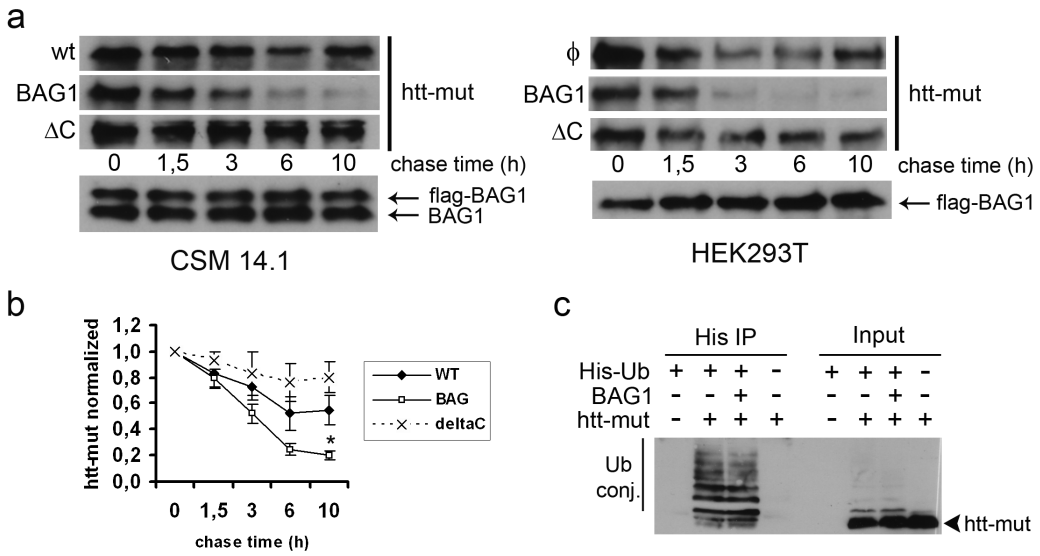



Fig. 4.9 BAG1 enhances htt-mut degradation.
CSM 14.1 wt, BAG1 or BAGΔC (ΔC) cells were transiently transfected with htt-mut. ~17 hours post-transfection, cycloheximide (chx) was added at 10 µg/ml for indicated time periods. Htt-mut is degraded faster in BAG1 cells, as compared to wild type cells. The presence of BAGΔC stabilizes htt-mut (a, left panel). Transient co-expression of htt-mut together with mock empty vector (ϕ), BAG1 or BAGΔC construct in HEK293T cells confirms the result obtained in CSM 14.1 cells (a, right panel). Endogenous BAG1 as well as the overexpressed flag-BAG1 levels do not decrease over the time course of the chx chase (a). Three independent experiments were quantified by densitometry (b, ANOVA, *, $p<0.05$; CSM 14.1 cells). HEK293T cells were transfected with htt-mut, BAG1 and His-ubiquitin (His-Ub) and subjected to nickel beads pull-down (His IP), followed by immunoblotting. The membrane was stained with an antibody against the polyQ stretch, revealing that BAG1 does not affect ubiquitination of htt-mut (c). Ub conj., ubiquitin conjugates.

BAG1 itself, on the other hand, appears to be a stable protein with its levels steady over the time course of the chase experiment. Interestingly, in the presence of BAGΔC htt-mut seemed to be even more stable (half-life ~9,5 hours) than in wild type cells, what correlates with previous results from our laboratory where BAGΔC appeared to have a dominant negative effect on protein folding (Liman et al., 2005). Again, we obtained identical results on huntingtin turn-over in transiently transfected HEK293T cells (4.9a, right). To investigate whether BAG1 changes the ubiquitination levels of mutant huntingtin, we transfected HEK293T cells with htt-mut, His-tagged ubiquitin and BAG1 or mock empty vector and performed affinity precipitation with nickel

beads. The beads bind to the His tag on ubiquitin and enable to precipitate free ubiquitin as well as all ubiquitinated proteins. In immunoblotting against htt-mut we observed a typical ladder formed by htt-mut with covalently attached ubiquitin chains of different length (Fig. 4.9c). The intensity of the staining did not change with addition of BAG1, meaning that it does not affect htt-mut ubiquitination and the enhanced degradation of htt-mut is mediated through a different mechanism.

## 4.4. BAG1 is protective in a cell culture model of HD

Enhanced degradation of mutant huntingtin together with its decreased aggregation and nuclear translocation in the presence of BAG1 suggested that BAG1 may be neuroprotective in HD. To test this hypothesis, we expressed BAG1 in striatal huntingtin knock-in cell lines, $STHdh^{+}$ and $STHdh^{Q111}$ (Trettel et al., 2000). These cell lines were developed by immortalization of embryonic striatal neurons derived either from wild type or transgenic mice, where an expanded polyglutamine chain was knocked into the murine huntingtin homologue gene (*Hdh*) (Wheeler et al., 2000). The cells were immortalized by transfection with temperature sensitive tsA58 SV40 large T antigen and are maintained at the permissive temperature of 32°C (Cattaneo and Conti, 1998). Once transferred to the non-permissive temperature of 39°C, the SV40 tsA58 protein is degraded and cells cease to proliferate with increased susceptibility to mutant huntingtin toxicity. We stably transfected these cell lines with BAG1 or a mock empty vector and tested whether overexpression of BAG1 can change their susceptibility to cell death at 39°C. Seven days after shifting the cells from 32°C to 39°C, $STHdh^{Q111}$ but not $STHdh^{+}$ cells show a 50% decrease in viability. $STHdh^{Q111}$ cells stably expressing BAG1 were protected from cell death, showing resistance similar to wild type $STHdh^{+}$ cells (Fig. 4.10).

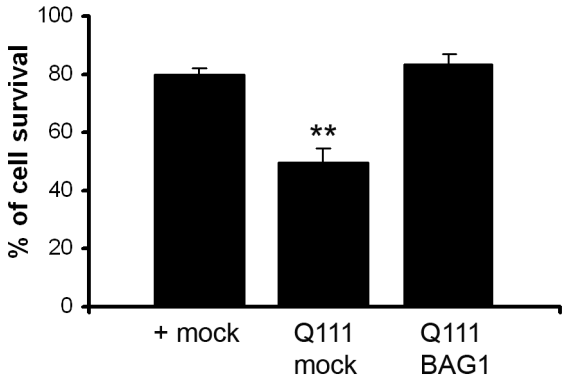



Fig. 4.10 BAG1 protects striatal cells from death induced by mutant huntingtin toxicity. STHdh$^{+}$ and STHdh$^{Q111}$ were stably transfected with BAG1 or a control empty vector (mock). Following shift to non-permissive temperature (39°C), the cells cease to proliferate. STHdh$^{Q111}$ cells are then vulnerable to mutant huntingtin (Q111) toxicity. After 7 days at 39°C, mock transfected STHdh$^{Q111}$ show 50% cell loss, which is prevented by stable expression of BAG1. The data represent mean values and SEM of three independent experiments (ANOVA, **, $p<0.01$).

## 4.5. BAG1 is protective in a *D. melanogaster* model of HD

We were then interested whether BAG1 displays any relevance for HD pathology *in vivo*. To this end, we employed a *D. melanogaster* model of HD, where expression of exon1 of mutant huntingtin with 93 glutamines (httQ93) leads to degeneration of the eye (Steffan et al., 2001). The compound eye of the fly consists of a regular array of hexagonal ommatidia. In wild type animals each ommatidium has 8 photoreceptor cells (7 of them visible) called rhabdomeres, arranged in a stereotypical trapezoid (Ready et al., 1976; Pichaud and Desplan, 2001). Flies expressing exon 1 of mutant huntingtin in all neuronal cells display a progressive degenerative phenotype manifested as a loss of rhabdomeres and their regular structure within an ommatidium (Fig. 4.11), which can serve as an assessment of huntingtin toxicity (Jackson et al., 1998).

We created BAG1 transgenic flies and crossed them with httQ93 flies, taking advantage of the UAS-GAL4 system which enables expression of the gene of choice in the tissue of interest of the fly. The target gene is cloned

downstream of a UAS (upstream activation sequence), which is a target sequence of the yeast transcription factor GAL4. The transgenic fly is then crossed to a fly having the *GAL4* gene downstream of an endogenous tissue-specific fly promoter. The endogenous promoter drives the expression of GAL4, which in turn drives the expression of the transgene (Phelps and Brand, 1998). Since the system requires to cross two different transgenic lines, and for our experiment we needed to combine three (elav-GAL4, httQ93 and BAG1), it was necessary to recombine BAG1 flies with elav-GAL4 flies, so that both transgenes are located on the same chromosome.

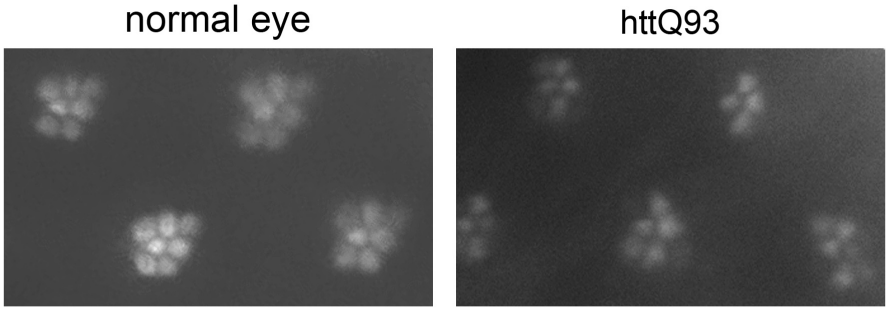



Fig. 4.11 Expression of exon1 of mutant huntingtin (httQ93) in neuronal cells of *D. melanogaster* leads to degeneration in the fly eye. Ten days post-eclosion, control flies have 7 regularly arranged rhabdomeres (photoreceptor cells) per each ommatidium. Expression of httQ93 under the control of the *elav-GAL4* driver leads to a reduction in rhabdomere numbers.

At day 10 following eclosion, httQ93 flies show a marked loss of rhabdomere numbers and disruption of their pattern which was visibly alleviated when mutant huntingtin was co-expressed with BAG1, as double transgenic flies had higher numbers of rhabdomeres per ommatidium and their organization resembled that of control flies (Fig 4.12a,b). To assure that this protective effect was not due to titration of the driver protein GAL4, which would then lead to a decrease in the amount of both transcripts by half and reduce huntingtin toxicity, we checked the expression levels of BAG1 by western blotting, comparing BAG1 flies to those co-expressing BAG1 with httQ93 and found no difference (Fig. 4.12c). This indicates that the alleviation of httQ93 toxicity was not due to decreased expression levels of transgenic proteins but a direct effect of BAG1.

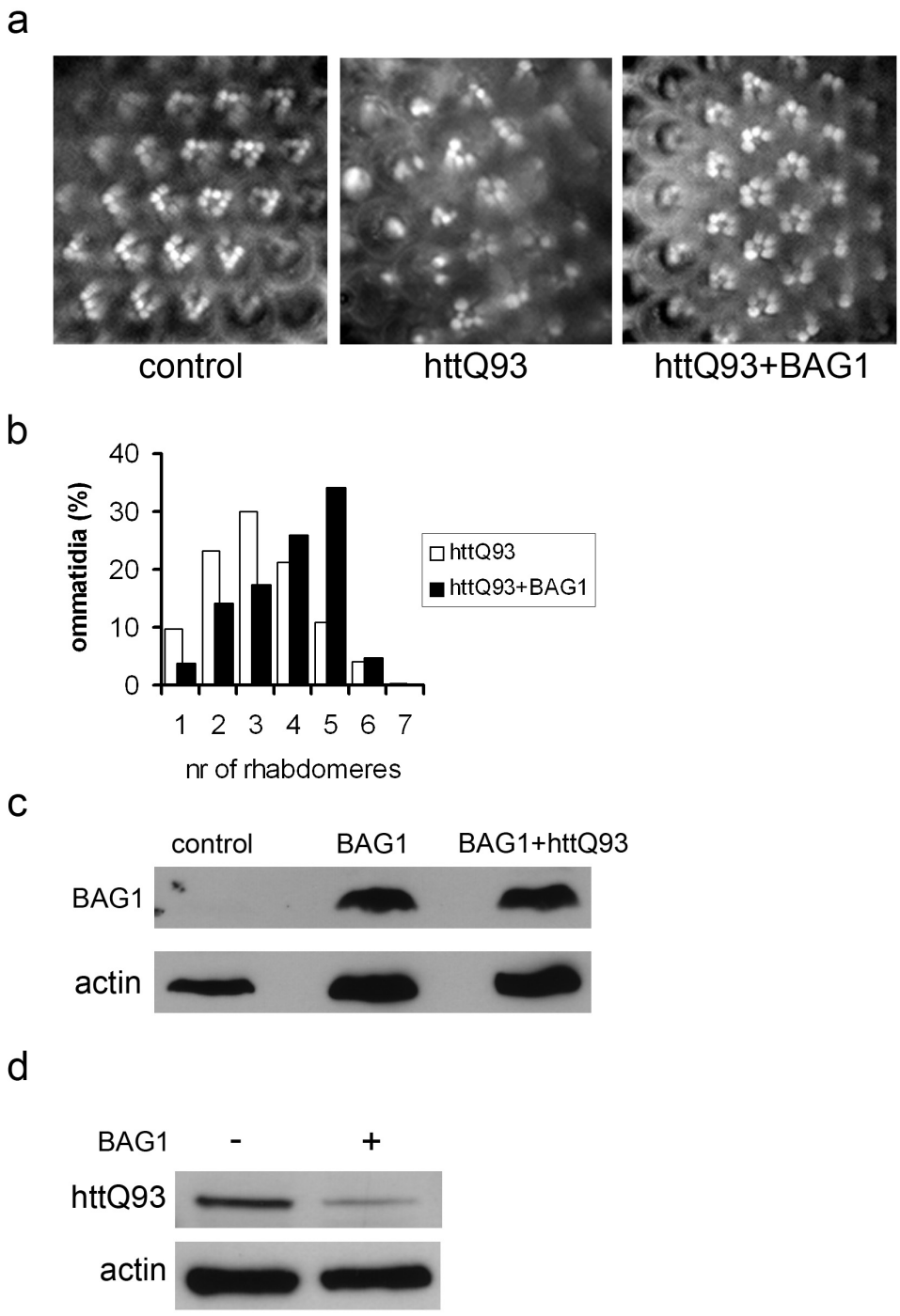



Fig. 4.12 BAG1 protects from httQ93 toxicity in *D. melanogaster* and reduces httQ93 protein levels.

The flies were analyzed at day 10 post-eclosion. Expression of httQ93 under the control of the pan-neuronal driver *elav-GAL4* leads to degeneration of the fly eye as seen by rhabdomere loss and disruption of their regular structure. Double transgenic flies, expressing BAG1 together with httQ93, are substantially protected from httQ93 toxicity. Representative photographs of ommatidia from control (*elav-GAL4*), httQ93 and httQ93/BAG1 flies (a). Quantification of the number of rhabdomeres per ommatidium in httQ93 and httQ93/BAG1 flies (b, at least 200 ommatidia were scored for each condition). The expression levels of BAG1 protein in fly heads of BAG1 flies and BAG1/httQ93 flies are the same (c). However, double transgenic flies have lower httQ93 levels compared to flies expressing httQ93 alone (d).

Since we observed that BAG1 decreases the half-life of htt-mut in cell culture, we wanted to check if this is the case in flies as well. We found that the expression level of httQ93 is lower in the presence of BAG1 (Fig. 4.12d). This suggests that the protective mechanism of BAG1 in this fly model of HD might be mediated via proteasomal degradation of the toxic protein.

To confirm our results, we performed another experiment using a different strain of flies with the *elav-GAL4* driver located on the first chromosome and employing a strategy to by-pass the recombination step, which in itself might create artefacts (Fig. 4.13). As an additional control we chose eGFP transgenic flies. Compared to the previous cross, in which *elav-GAL4* was on the third chromosome, the httQ93 phenotype is milder when induced by *elav-GAL4* on the first chromosome. Also with this approach BAG1 ameliorates httQ93-induced photoreceptor cell loss (Fig. 4.14). In contrast to BAG1/httQ93 flies, those expressing eGFP together with httQ93 were not protected from toxicity, as revealed by rhabdomere counting, confirming BAG1 specific conservation of the eye structure.

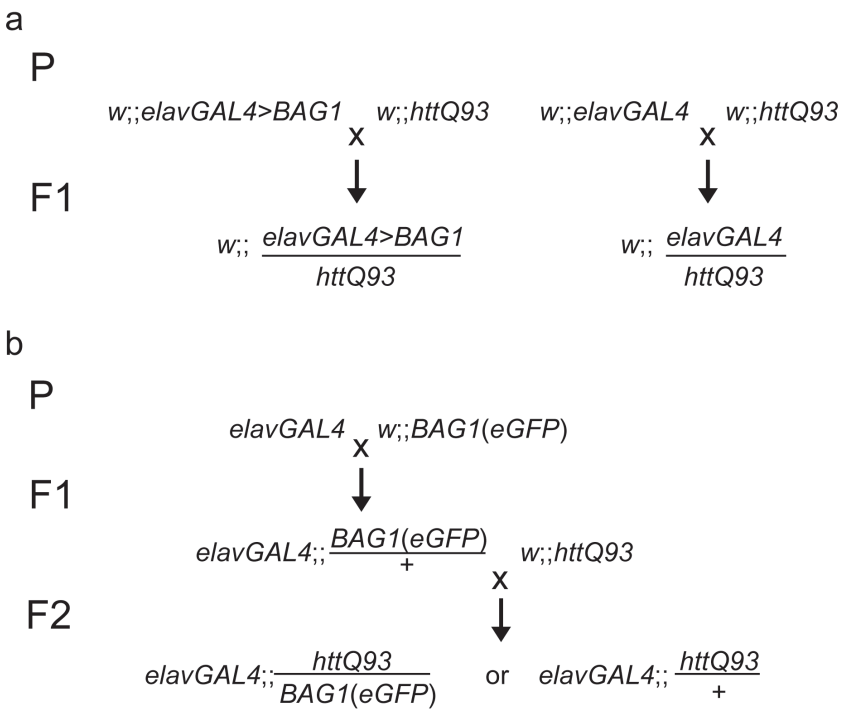



Fig. 4.13 Two crossing schemes used to generate double transgenic flies (a, b). Chromosomes are separated by semicolons. P- parental generation, F1, F2- first and second filial generations, + indicates a wild type chromosome, w, “white” gene.

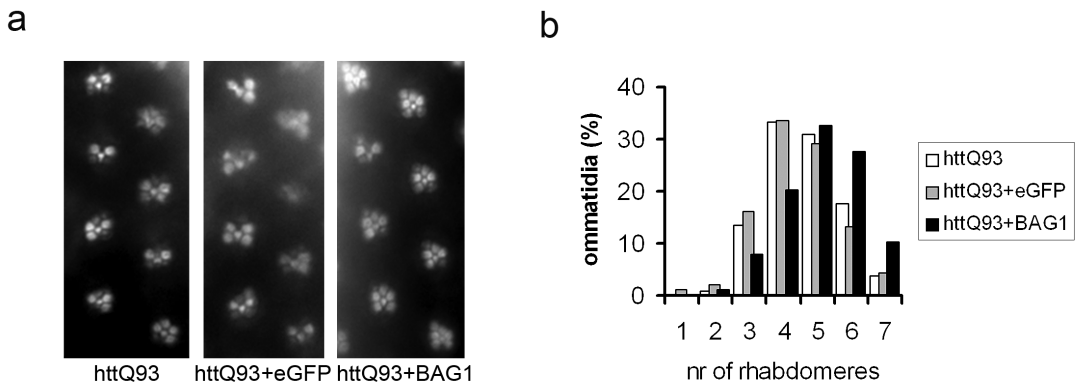



Fig. 4.14 BAG1, but not eGFP, protects from httQ93-induced eye degeneration in *D.melanogaster*.

Representative photographs of ommatidia from httQ93, httQ93/eGFP and httQ93/BAG1 flies 10 days post-eclosion (a). The loss of rhabdomeres in flies expressing httQ93 is ameliorated by co-expression of BAG1. Co-expression of eGFP does not mitigate the httQ93 phenotype (b, at least 200 ommatidia were scored for each condition).

# 5. DISCUSSION

This study was performed to evaluate the potentially beneficial effect of the co-chaperone BAG1 in various paradigms of mutant huntingtin toxicity. Moreover, it was the focus of this project to unravel the mechanisms underlying its putative effect. BAG1 is a multifunctional protein critical for the nervous system development and regeneration (Kermer et al., 2002; Kermer et al., 2003; Gotz et al., 2005). It provides a link between cell cycle, apoptosis, stress response and protein degradation (Takayama et al., 1995; Demand et al., 2001; Song et al., 2001). An interaction with mutant huntingtin fragment has already been suggested (Jana and Nukina, 2005). However, the relevance of BAG1 for HD pathophysiology and its mechanism of action have not yet been elucidated.

## 5.1. Nuclear localization of mutant huntingtin

We discovered that BAG1 substantially decreases the amount of mutant huntingtin in the nucleus. This appears important considering the evidence that nuclear translocation of huntingtin is a critical event for its cytotoxicity (Saudou et al., 1998). Adding a nuclear localization signal to mutant huntingtin fragment leads to increased toxicity, while a nuclear export signal decreases it in cell culture (Peters et al., 1999). Likewise, nuclear targeting of mutant huntingtin in mice exacerbates their phenotype (Benn et al., 2005). It is thought that the presence of mutant huntingtin fragment in the nucleus can disrupt the transcriptional profile of striatal neurons through interactions with transcription factors (Sugars and Rubinsztein, 2003; Thomas, 2006; Truant et al., 2007). On the other hand, the presence of mutant huntingtin in the cytosol undoubtedly contributes to its toxicity by influencing axonal transport, protein folding, synaptic transmission and mitochondrial function (Muchowski, 2002; Gunawardena et al., 2003; Szebenyi et al., 2003; Browne and Beal, 2004; Smith et al., 2005; Gidalevitz et al., 2006). Nonetheless, it is a well established fact that nuclear translocation is crucial for the disease pathology. Decreasing

the amount of mutant huntingtin in the nucleus could therefore provide a promising therapeutic strategy.

Investigating how BAG1 actually decreases htt-mut nuclear content, we found that blocking the proteasome by a pharmacological inhibitor abolishes this effect. In this regard, BAG1 has already been implicated in the proteasomal pathway as a protein that binds the proteasome through its ubiquitin-like domain (Luders et al., 2000; Alberti et al., 2002) and targets substrate proteins for degradation (Demand et al., 2001). Hence, our observation of increased huntingtin turn-over in the presence of BAG1 is plausible.The degradation could occur either directly in the nucleus or in the cytoplasm, before the mutant protein is transported to the nucleus, or both.

Alternatively, BAG1 could act by changing htt-mut shuttling between the cytoplasm and the nucleus. The mechanisms by which huntingtin is transported to and from the nucleus still remain obscure. Huntingtin has a conserved C-terminal nuclear export signal (Fig. 1.1) which is cleaved away when it is processed by cellular proteases (Xia et al., 2003). Short N-terminal fragments that are pathogenic in HD models are probably small enough to pass through the nuclear pore by passive diffusion (Stewart et al., 2001). Nevertheless, abnormal accumulation of mutant huntingtin in the nucleus suggests that there is an active component involved. It has been shown that the N-terminus of huntingtin interacts with nuclear pore protein TPR (translocated promoter region) which is involved in nuclear export and that polyglutamine chain expansion decreases this interaction, leading to nuclear accumulation of mutant huntingtin (Cornett et al., 2005). Regarding nuclear import, it was recently reported that Siah1 is involved in this process. Siah1 is a proapoptotic E3-type ubiquitin ligase activated by the p53 transcription factor (Amson et al., 1996; Hu and Fearon, 1999; Roperch et al., 1999; Reed and Ely, 2002). It contains a nuclear localization signal and was found to form a complex together with mutant huntingtin fragment and GAPDH, which then translocates to the

nucleus and induces cell death (Fig. 4.4) (Bae et al., 2005; Hara et al., 2005). BAG1 is known to bind and inhibit Siah1, and it could decrease htt-mut nuclear content by counteracting the effect of Siah1 (Matsuzawa et al., 1998). Siah1 is an unstable protein which self-ubiquitinates resulting in its degradation by the proteasome. To overcome this experimental limitation, we created a deletion mutant of Siah1 lacking the RING domain necessary for its ubiquitin ligase activity (Freemont, 2000). Using this mutant we found that overexpression of Siah1 overcomes the effect of BAG1 on htt-mut subcellular localization. Also, BAG1 decreases both the overexpressed Siah1ΔRING as well as the endogenous Siah1 levels. Thus, our results suggest that BAG1 could reduce htt-mut localization by three distinct mechanisms:

- targeting of htt-mut for degradation via the UPS
- binding and inhibiting Siah1
- targeting of Siah1 for degradation via the UPS

Nevertheless, it remains difficult to discern between these three options. Proteasomal inhibition obviously affects the expression levels of both htt-mut and Siah1. One could use a BAG1 N-terminal deletion mutant which retains the ability to bind to Siah1 *in vitro* but lacks its UBL domain and is, therefore, unlikely to still play a role in substrate delivery to the proteasome (Matsuzawa et al., 1998). Unfortunately, attempts to create such a mutant were unsuccessful and resulted in a very unstable protein in our hands. An alternative solution would be to search for a point mutant of BAG1 that does not bind the proteasome. Another BAG1 mutant that was employed in our study, BAGΔC lacking the C-terminal BAG domain, did not prevent nuclear accumulation of htt-mut. In line with our hypothesis, this mutant does not interact with Siah1 anymore (Matsuzawa et al., 1998). However, the deletion also abolishes its binding to Hsp70 (Takayama et al., 1997) and mutant huntingtin fragment (Jana and Nukina, 2005). One cannot, therefore, draw straightforward conclusions since lack of activity of BAGΔC could result from disruption of any of the interactions listed above.

## 5.2. Mutant huntingtin aggregation is decreased in the presence of BAG1

We observed that BAG1 overexpression leads to a decrease in the amount of htt-mut aggregation as revealed by counting single cells containing inclusion bodies and a biochemical filter retardation assay. Furthermore, BAG1 colocalized with htt-mut_eGFP inclusion bodies or was found in a rim surrounding them. This was not particularly surprising since many components of the chaperone system were found in misfolded proteins inclusion bodies and could influence their aggregation [see “Chaperone mediated neuroprotection” in the Introduction and (Muchowski and Wacker, 2005)]. Originally, aggregation was considered to be a random process resulting from unspecific interactions of non-native proteins that have a tendency to “stick together” (Lansbury, 1997). Recently, it has become evident that protein aggregation is a very specific phenomenon driven not solely by the inherent tendency of misfolded proteins to agglomerate but that it is a regulated process through which the cell tries to sequester deleterious proteins as a protection mechanism (Johnston et al., 1998; Garcia-Mata et al., 1999; Rajan et al., 2001). Expression of a mutant protein or inhibition of the proteasome leads to an accumulation of misfolded ubiquitinated proteins. They form microaggregates that are then transported along microtubules to the microtubule organizing centre (MTOC), where they form one big inclusion termed aggresome (Kopito, 2000; Muchowski et al., 2002). Inside the nucleus, aggregates are also localized to a specific subcompartment called promyelocytic leukemia (PML) body (Anton et al., 1999; Takahashi et al., 2003). The formation of aggresomes is an active process that requires intact microtubule skeleton, chaperones and the UPS components. It is assumed that inclusion body formation is a way the cell tries to separate toxic proteins in a compartment serving as a temporary “storage room” until non-native peptides can be refolded or degraded. It was shown that cells have ways to efficiently remove inclusions once the proteasomal impairment or the supply of mutant protein ceases (Anton et al., 1999; Yamamoto et al., 2000). In our cellular system we observed that overexpression of eGFP tagged htt-

mut results in formation of multiple small aggregates in the cytoplasm and the nucleus which with time grew bigger and frequently formed a single aggresome-like inclusion in the vicinity of the nucleus. The presence of BAG1 in those inclusions suggests that it is a part of the chaperone response activated in order to remove the toxic protein. Trying to elucidate the mechanism of how BAG1 reduces the amount of htt-mut aggregation, we used its mutant lacking the C-terminal Hsp70 binding BAG domain (BAGΔC) and documented that it does not protect from inclusion body formation. This could indicate that BAG1 can assist Hsp70 in refolding of htt-mut. On the other hand, we also observed that blocking of the proteasome completely abolishes the effect BAG1 has on htt-mut aggregation, suggesting that BAG1 might promote htt-mut degradation. That way the mutant protein would be removed before it is able to accumulate and form inclusion bodies.

### 5.3. BAG1 stimulates mutant huntingtin degradation

Showing that BAG1 influence on htt-mut subcellular localization and aggregation can be overcome by inhibition of the proteasome and that BAG1 can associate with the proteasome, we sought to find out whether it directly affects htt-mut degradation. In a cycloheximide chase experiment we observed that htt-mut is removed faster in the presence of excess BAG1, although its ubiquitination levels are unaffected. BAG1 itself appears to be quite stable, what correlates with a report that BAG1 association with the proteasome does not result in its degradation (Alberti et al., 2002). BAG1 was previously shown to enhance Hsp70 association with the proteasome, accept ubiquitinated glucocorticoid hormone receptor from Hsp70 and enhance its degradation, acting together with CHIP ubiquitin ligase (Luders et al., 2000; Demand et al., 2001). BAG1 binds to Hsc70 and leads to a conformational change in its ATPase domain, which results in substrate release (Gassler et al., 2001; Sondermann et al., 2001). Here, we show for the first time that overexpression of BAG1 alone can lead to faster degradation of a misfolded protein. It is

plausible that htt-mut is first recognized and bound by a chaperone of the Hsp70 family. Binding of BAG1 to Hsp70 targets htt-mut to the proteasomal complex and induces its release, so that it can be degraded.

Several components of the UPS, E3 ubiquitin ligases in particular, were shown before to be able to stimulate degradation of polyQ expansion proteins and reduce their toxicity (Jana et al., 2005; Al-Ramahi et al., 2006; Yang et al., 2007; Mishra et al., 2008). The example of BAG1 is the first to show enhanced degradation of htt-mut by a different mechanism than increase in ubiquitination, i.e. targeting to the proteasome.

Intriguingly, expression of BAGΔC mutant, which still contains the UBL domain that mediates proteasomal association but is unable to bind to Hsp70, results in stabilization of htt-mut. This is reminiscent of the previously reported dominant negative effect of BAGΔC mutant on folding of a fluorescent chaperone sensor (Liman et al., 2005). The inability of BAGΔC to degrade htt-mut would suggest that BAG1 acts in a complex that presumably involves a chaperone of the Hsp70 family to efficiently target substrates to the proteasome. However, why would the expression of BAGΔC have a negative effect on mutant huntingtin fragment degradation? Since it still binds to the proteasome, one possible explanation is a physical obstruction of access to the “docking station” for the chaperone complex.

## 5.4. BAG1 protects from mutant huntingtin toxicity *in vitro* and *in vivo*

It is well accepted that mutant huntingtin must translocate to the nucleus in order to induce cell death (Saudou et al., 1998), and we observed that BAG1 can reduce htt-mut nuclear levels. This, together with the ability of BAG1 to promote htt-mut degradation, suggested that BAG1 could ameliorate mutant huntingtin toxicity. We confirmed this hypothesis using a cell culture and a *D. melanogaster* model of HD. The cellular model we used originates from striatal

neurons isolated from genetically engineered mice in which an expanded polyQ chain was knocked-in to the murine huntingtin homologue gene (Trettel et al., 2000). It, therefore, expresses full-length huntingtin as opposed to the truncated fragment we used for the previous experiments. We established stably transfected lines expressing BAG1 and documented that it can rescue the cells from toxicity induced by mutant huntingtin. We confirmed this result *in vivo,* for which we chose *D. melanogaster* as a model organism. The expression of polyQ expanded proteins in the nervous system of *Drosophila* leads to a neurodegenerative phenotype in the eye, which can be evaluated using a simple imaging method. We generated BAG1 transgenic flies and crossed them with flies expressing exon1 of mutant huntingtin (httQ93). As hypothesized, BAG1, but not eGFP control, leads to a marked improvement of the eye structure. Furthermore, BAG1 seems to decrease the amount of httQ93 on the protein level suggesting that BAG1 neuroprotection is mediated by its ability to stimulate mutant huntingtin degradation also *in vivo*. This would reconcile the effect BAG1 has on htt-mut aggregation with its neuroprotective function. Inclusion body formation has been shown to actually promote striatal cell survival, and the levels of diffuse soluble huntingtin correlate with cell death (Arrasate et al., 2004). However, it appears that BAG1 reduction of inclusion body formation is mediated through its ability to target the mutant protein for degradation, thereby preventing htt-mut aggregation by removing the monomeric species rather than resolubilizing already existing aggregates.

## 5.5. Potential therapeutic implications

It is clear from numerous studies conducted in recent years that stimulation of the protein quality control system, which comprises chaperones and the degradation machinery, is so far the most potent strategy of suppressing neurodegeneration in laboratory models of human diseases. Accumulation of misfolded proteins underlies several neurodegenerative disorders, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and other

polyQ expansion diseases. Although they all affect different populations of neurons and lead to toxicity through distinct mechanism, they all share the feature of abnormally folded proteins that form ubiquitinated inclusions. It is thought that protein misfolding is particularly harmful for neuronal cells since they are unable to dilute the load of toxic proteins through cell division. Additionally, it was observed that senescent cell cultures and aged organisms as well as human tissue have a reduced ability to activate heat shock proteins in response to various stressful stimuli (Rattan and Derventzi, 1991; Heydari et al., 1994; Locke and Tanguay, 1996; Muramatsu et al., 1996). Also, the protein degradation systems, the lysosome pathway and the ubiquitin-proteasome pathway, have a reduced capacity associated with aging (Shang et al., 1997; Cuervo and Dice, 1998). The functional decline of the protein quality control mechanisms seen in aging might account for the late onset (middle age or later) of the above mentioned neurodegenerative diseases. It appears that for many years neurons are actually able to deal with misfolded proteins until their defenses are weakened by age, and the toxic protein accumulates above a critical threshold. Thus, stimulating the chaperone machinery and the degradation pathway seem to be a promising therapeutic avenue.

Here, we present the multifunctional protein BAG1 as a protective therapeutic strategy in HD models acting by association with Hsp70 to stimulate the degradation of mutant huntingtin fragment via the UPS. Furthermore, we uncovered inhibition of Siah1 as an alternative way - besides increased proteasomal turn-over - through which BAG1 could decrease the amount of mutant huntingtin in the nucleus and protect from mutant huntingtin toxicity. What makes BAG1 a particularly interesting therapeutic target is the fact that in addition to promoting degradation of misfolded proteins it can also prevent cell death (Takayama et al., 1995; Danen-van Oorschot et al., 1997; Kermer et al., 2002). Furthermore, BAG1 is an established activator of the Raf-1 signaling pathway, which acting through ERK, results in synaptic plasticity, long term potentiation and cell survival in response to stressful stimuli (Grewal et al.,

1999). Recent observations in our laboratory indicate that BAG1 is a potent inducer of axonal outgrowth after axotomy (Planchamp et al., in revision). This quality is particularly important with regard to diseases affecting the nervous system, where preservation of the cell body is not sufficient to maintain the function of neurons and treatments that combine cell death prevention and axonal regeneration would be most valuable.

However, as much as BAG1 and other components of the protein quality control system seem to be promising therapeutic targets, one has to be aware of the possible side effects of such potential treatments. All proteins that affect cell cycle and prevent apoptosis might induce malignancy, and BAG1 is indeed found over-expressed in some cancers (Cutress et al., 2002; Mosser and Morimoto, 2004; Dahlmann, 2007). Further studies leading to a more detailed understanding of the protein quality control in neurodegenerative disorders and a delicate balance of chaperones will be necessary to achieve a desired therapeutic effect.

# 6. SUMMARY

Huntington's disease (HD) is a fatal neurodegenerative disorder resulting from an expansion of a polyglutamine stretch in a ubiquitously expressed protein called huntingtin. Intraneuronal inclusion body formation and accumulation of mutant huntingtin N-terminal fragments in the nucleus are the pathological hallmarks of the disease. It is thought that nuclear localization of mutant huntingtin leads to toxicity, while inclusion body formation exerts a beneficial function of sequestering the toxic fragment.

We evaluated the effect of the co-chaperone BAG1 on mutant huntingtin toxicity and investigated its mechanism of action. BAG1 is a multifunctional protein, delivering chaperone-recognized substrates to the proteasome for degradation. It is essential for proper CNS development, it stimulates neuronal differentiation and protects from a variety of apoptotic insults. Here, we show that BAG1 reduces inclusion body formation and accelerates the degradation of mutant huntingtin in a proteasome- and Hsp70-dependent manner. Moreover, it inhibits translocation of mutant huntingtin into the nucleus. This effect can be overcome by overexpression of Siah-1, an E3-ligase negatively regulated by BAG1 and known to be involved in nuclear import of mutant huntingtin. In addition, we observed that BAG1 leads to decreased cellular levels of Siah1. Finally, BAG1 protects from mutant huntingtin-induced cell death *in vitro* and *in vivo*. In a *Drosophila* HD model BAG1 significantly ameliorates photoreceptor cell loss and leads to decreased levels of mutant huntingtin fragment. In summary, we present BAG1 as a novel therapeutic tool modulating key steps of huntingtin toxicity *in vitro* and ameliorating huntingtin toxicity *in vivo*.

# 7. ABBREVIATIONS

**A** adenine
**aa** amino acid
**AAA** ATPases associated with a variety of cellular activities
**ADP** adenosine diphosphate
**Atg** autophagy-related gene
**ATP** adenosine 5'-triphosphate
**BAG1** Bcl-2 associated athanogene
**Bcl-2** B cell lymphoma 2
**BDNF** brain derived neurotrophic factor
**BSA** bovine serum albumin
**C** cytosine
°**C** degree Celsius
**CCT** cytosolic chaperonin
**Cdk5** cyclin-dependent kinase 5
**cDNA** complementary DNA
**C. elegans** Caenorhabditis elegans
**CHIP** carboxyl terminus of Hsc70-interacting protein
**chx** cycloheximide
**CNS** central nervous system
**CREB** cAMP response element-binding
**C-terminus** carboxy-terminus
**DAPI** 4',6-diamidino-2-phenylindole
**dATP** deoxyadenosine triphosphate
**dCTP** deoxycytidine triphosphate
**dGTP** deoxyguanosine triphosphate
**dNTP** deoxyribonucleotide triphosphate
**dTTP** deoxythymidine triphosphate
**D. melanogaster** Drosophila melanogaster
**DMEM** Dulbecco's Modified Eagle's Medium
**DMSO** dimethyl sulfoxide
**DNA** deoxyribonucleic acid
**E6-AP** E6-associated protein
**ECL** enhanced chemiluminescence
**E.coli** Escherichia coli
**EDTA** ethylenediaminetetraacetic acid
**eGFP** enhanced green fluorescent protein

***elav*** embryonic lethal, abnormal vision
**ER** endoplasmic reticulum
**ERK** extracellular signal-regulated kinase
**FBS** foetal bovine serum
**G** guanine
**GAPDH** glyceraldehyde 3-phosphate dehydrogenase
**h** hour
**H2B** histone 2B
**HD** Huntington's disease
**Hdh** Huntington's disease gene homologue
**HEK** human embryonic kidney
**HIP1** huntingtin interacting protein 1
**HIPPI** HIP1 protein interactor
**HRP** horseradish peroxidase
**Hsp** heat shock protein
**htt** huntingtin
**htt-mut** mutant huntingtin
**htt-wt** wild type huntingtin
**JNK** c-Jun N-terminal kinase
**kDa** kilodalton
**l** liter
**LB** Luria Bertani
**Lys** lysine
**M** mole
**m** milli
**MAP** mitogen-activated protein
**MAPK** mitogen-activated protein kinase
**MDa** megadalton
**MEK** MAP/ERK kinase
**MEKK** MEK kinase
**min** minute
**MKK** MAP kinase kinase
**MTOC** microtubule organizing centre
**n** nano
**µl** micro
**mTOR** mammalian target of rapamycin
**NII** neuronal intranuclear inclusion
**Ni-NTA** nickel nitrilo-triacetic acid

**NMDA** N-methyl-D-aspartic acid
**NRSF** neuronal restrictive silencing factor
**N-terminus** amino-terminus
**OD** optical density
**P** proline
**PBS** phosphate buffered saline
**PCR** polymerase chain reaction
**PGC-1**α peroxisome proliferator-activated receptor-γ coactivator 1α
**PFA** paraformaldehyde
**PI3K** phosphoinositide-3 kinase
**PIPES** 1,4-piperazinediethanesulfonic acid
**PML** promyelocytic leukemia
**PSD-95** postsynaptic density-95
**Q** glutamine
**REST** RE1-silencing transcription factor
**RING** really interesting new gene
**RIPA** radioimmuno precipitation assay
**RNA** ribonucleic acid
**RT-PCR** reverse transcription PCR
**SAPK** stress-activated protein kinase
**sec** second
**SEM** standard error of the mean
**Siah1** seven in absentia homologue 1
**SDS** sodium dodecyl sulphate
**SDS-PAGE** sodium dodecyl sulphate polyacrylamide gel electrophoresis
**TBE** Tris-Borate-EDTA
**TBP** TATA-binding protein
**TBS** Tris-buffered saline
**TEMED** tetramethylethylenediamine
**TPR** translocated promoter region
**TRiC** tail-less complex polypeptide 1 ring complex
**Tris** trishydroxymethylaminomethane
**TFIID** transcription factor II D
**UAS** upstream activating sequence
**UBL** ubiquitin-like
**UPS** ubiquitin-proteasome system
**V** volt
**v/v** volume/volume percentage
**VCP** valosin-containing protein

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# 9. CURRICULUM VITAE

Personal details:

Name: Kamila Sroka
Nationality: Polish
Date and place of birth: 14.10.1981 in Lublin, Poland

Education:

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|---|---|
| November 2004- March 2008 | PhD candidate in the Neurobiological Research Laboratory, University Hospital Göttingen, Germany |
| October 2003- September 2004 | International Max Planck Research School for Neurosciences, Göttingen, Germany |
| October 2000- June 2003 | B.Sc. in Biotechnology Jagiellonian University, Krakow, Poland |

Scholarships:

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|---|---|
| 2004- 2007 | Neuroscience Early Stage Research Training (NEUREST) Marie Curie Actions Fellowship |
| 2003-2004 | International Max Planck Research School Stipend |

Scientific courses:

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|---|---|
| 6 December 2005 | Scientific Publishing, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany |
| 21-22 August 2006 | Working with protein structures. Computer-based data analysis, University of Göttingen, Germany |
| 18-19 June 2007 | Posttranslational modification with ubiquitin-related protein SUMO, University of Göttingen, Germany |

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|---|---|
| 14-15 August 2007 | Protein-protein interactions in membrane traffic<br>Max Planck Institute for Biophysical Chemistry, Göttingen, Germany |
| 3-14 September 2007 | The ubiquitin-proteasome system in health and disease<br>RUBICON Graduate Course, Karolinska Institutet, Stockholm, Sweden |

Conferences

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| 12-14 November 2004 | International PhD Student Symposium Neurizons<br>Göttingen, Germany |
| 7-10 May 2005 | II Meeting on the Molecular Mechanisms of Neurodegeneration<br>Milano, Italy |
| 27-28 May 2005 | Parkinson's disease and other neurodegenerative disorders: from genes to molecular mechanisms and therapy<br>Göttingen, Germany |
| 9-12 February 2006 | NeuroNE First Annual Meeting<br>London, UK |
| 25-30 June 2006 | The 18th Meeting of Nobel Laureates in Chemistry<br>Lindau, Germany |
| 14-18 October 2006 | Sfn Neuroscience 2006 Conference<br>Poster contribution<br>Atlanta, USA |
| 29 March-1 April 2007 | 7th Meeting of the German Neuroscience Society, 31st Göttingen Neurobiology Conference<br>Poster contribution<br>Göttingen, Germany |
| 31 May-2 June 2007 | International PhD Student Symposium Neurizons 2007 |

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| Miscellaneous: | Member of the organization committee of Neurizons 2007 conference |

# 10. PUBLICATIONS

**Sroka, K.**, Voigt, A., Deeg, S., Reed, J.C., Schulz, J.B., Bähr, M., Kermer, P. "Huntingtin toxicity is ameliorated by BAG1 through modulation of its aggregation, degradation and subcellular distribution." submitted

Ganesan, S., Rohde, G., Eckermann, K., **Sroka, K.**, Schaefer, M.K.E., Dohm, C.P., Kermer, P., Haase, G., Wouters, F., Bähr, M. and Weishaupt, J.H. (2008)
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