Activated caspase-6 and caspase-6-cleaved fragments of huntingtin specifically colocalize in the nucleus

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INTRODUCTION

Huntington disease (HD) is an inherited neurodegenerative disorder that results from polyglutamine-expansion in the N-terminal of the mutant huntingtin protein (1,2). Despite the widespread expression of huntingtin, initial neuropathology is selective to medium spiny neurons of the striatum.

Huntingtin can be cleaved at many sites by caspsases (3–6), calpains (7,8) and aspartyl proteases (9). Multiple cleavage fragments of huntingtin are observed in YAC128 mice, which express full-length human mutant huntingtin and develop HD-like cognitive dysfunction, motor deficits and selective striatal degeneration in an age, expression-level and polyglutamine-length-dependent fashion (10–13).

Studies in mice that express caspase-resistant mutant huntingtin indicate that huntingtin proteolysis specifically at amino acid 586 is critical to the pathogenesis of the disease. Preventing huntingtin cleavage at amino acid 586 in the caspase-6-resistant (C6R-YAC128) mice prevents the development of motor, cognitive and neuropathological features of the disease phenotype (14). This protective effect is specific to amino acid 586 cleavage because in contrast, blocking proteolysis at the caspase-2/3 recognition sites, amino acid 552 and amino acid 513, in the caspase-3-resistant (C3R-YAC128) mice did not prevent the development of the features of HD as observed in the YAC128 mice (14). Why the toxicity of mutant huntingtin is specifically associated with the amino acid 586 cleavage event relative to caspase cleavage at other sites in huntingtin is not known.

Specific cleavage events are known to be important in other neurodegenerative diseases such as Alzheimer disease (AD) (15,16) and several spinocerebellar ataxias (17–19).
Importantly, preventing caspase cleavage of β-amyloid precursor protein (APP) prevents the behavioural and neuropathological features of AD (20).

Nuclear accumulation of mutant huntingtin is a marker of disease seen in human brain (21,22). In addition, in the YAC128 mouse, both the nuclear localization of huntingtin and neuropathology coincidently occur earliest and to the greatest extent in the lateral striatum relative to other brain regions (12,23). In comparison, the nuclear accumulation of huntingtin is significantly reduced and delayed in the C6R-YAC128 mice, which are protected from the toxic effects of mutant huntingtin (14).

Specific, unidentified soluble fragments of huntingtin in the nucleus are important for toxicity, rather than the presence of nuclear inclusions, since the size and number of nuclear inclusions does not correlate with cytotoxicity in cell models (24,25) or the shortstop mouse model of HD, where extensive inclusions are present but do not cause neurodegeneration (26). Studies from human lymphoblasts (27) and fibroblasts (28) have suggested that some huntingtin cleavage products are localized to the nucleus. However, which fragments of huntingtin are found in the nucleus and confer nuclear toxicity are unknown.

Excessive activation of Caspase-6 (C6, Mch2), a key caspase known to play both initiator and effector roles in programmed cell death, is an early marker of neuronal dysfunction and has been implicated in the pathogenesis of HD (14,29), AD (30–32) and other forms of cognitive impairment (33,34).

There are key unresolved questions that arise from studies of the C6R-YAC128 mice: What differentiates the C6 derived 586 amino acid huntingtin fragment from other huntingtin fragments and how does the 586 amino acid fragment mediate toxicity? How and where in the cell is C6 activated? Using neo-epitope antibodies specific to huntingtin when cleaved at specific caspase sites, we have examined the subcellular localization of huntingtin fragments and determined that endogenously generated huntingtin fragments have different and unique cellular distributions depending on the size of the fragment. Importantly, endogenously generated 586 amino acid fragments and active C6 are enriched and co-localize in the nucleus. This finding may provide insight as to why mutant huntingtin toxicity is specifically associated with the huntingtin 586 amino acid fragment.

RESULTS

Cleavage sites in huntingtin fall between HEAT domains

Sequence-based structural predictions of huntingtin suggest that the previously characterized caspase and calpain cleavage sites in huntingtin are clustered among several important structural features of the protein. Huntingtin is a large protein that is predominantly composed of HEAT repeats (35,36) that are clustered into four major HEAT domains (Fig. 1A). The current structural model of full length huntingtin is of a flexible superhelical solenoid composed of folded and stacked HEAT repeats with a continuous hydrophobic core (37). As with other HEAT repeat proteins, this structural model is consistent with huntingtin functioning as a scaffold protein.

Huntingtin also contains several PEST sequences, polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T), that are postulated to target proteins for proteolysis (38). Interestingly, three of the four predicted PEST domains are interspersed between HEAT domains. The previously characterized caspase and calpain recognition sites of huntingtin fall into PEST domain 2 (Fig. 1A), which is found in the intervening sequence between HEAT domains 1 and 2. This includes the recognition sequence at amino acid 586 (Fig. 1B) which is cleaved in vitro by C6 that, when mutated to prevent cleavage, prevents the HD phenotype otherwise seen in the YAC128 mouse model (14). These structural predictions are consistent with the hypothesis that proteolytic processing of huntingtin in PEST domain 2 has a function beyond simple degradation of the protein.

Characterization of the neo-htt586 (IVLD) antibody

In order to further investigate the generation and localization of the huntingtin amino acid 586 fragment, a 586-IVLD-epitope antibody (neo-htt586) was generated (Fig. 1C). IVLD-COO− is the amino-acid epitope that is exposed when huntingtin is cleaved specifically at amino acid 586. The IVLD cleavage site is unique to huntingtin, as no other IVLD substrates for C6 are known (Supplementary Material, Table S1). To demonstrate that the neo-htt586 antibody only detects huntingtin when cleaved at amino acid 586, immunoblotting (Fig. 1D) and immunofluorescent staining (Fig. 1E) were performed against discrete huntingtin caspase fragments of different lengths. Immunoblots from HEK293 cell lysates demonstrate that neo-htt586 specifically detects 586 amino acid fragments, and not longer (such as full-length huntingtin) or shorter (1–552 amino acid) huntingtin fragments (Fig. 1D). This is consistent with immunofluorescence data in transfected COS cells in which the neo-htt586 antibody stains cells expressing the truncated 1–586 amino acid fragment, but not huntingtin truncated at amino acid 552 (Fig. 1E) or other cleavage sites. In addition, the intensity of the neo-htt586 staining is positively correlated with the levels of htt in the cell (Supplementary Material, Fig. S2). In addition, the neo-htt552 and neo-htt513 antibodies, which are generated against epitopes that are exposed by huntingtin cleavage at amino acid 552 and amino acid 513, also specifically detect their corresponding fragments but not any other huntingtin fragments (27,39).

Subcellular localization of endogenously generated huntingtin caspase fragments is influenced by the size of the fragment

Neo-epitope staining in unstimulated cells reveals that endogenous caspase-cleaved huntingtin fragments have a markedly different subcellular localization depending on the amino acid position of proteolysis (Fig. 2A). In the untreated ST14 cell line (originally derived from the rat primordial striatum), neo-htt586 antibody staining was enriched in the nuclear compartment. Neo-htt552 staining was concentrated around the nucleus, and associated closely with the nuclear
Figure 1. The neo-htt586 antibody specifically detects huntingtin 586 amino acid fragments. (A) Structural predictions of the huntingtin protein place many previously characterized proteolysis sites in PEST domain 2, which falls between HEAT domains 1 and 2. Predicted HEAT repeats are clustered together in four HEAT domains, indicated by red barrels. The amino acid start and finish of each HEAT domain is indicated (see Supplementary Material, Fig. S1). (B) Amino acid sequence of huntingtin between amino acid 481 and amino acid 600 showing the caspase proteolysis sites (in blue) and silent caspase sites (in grey). (C) Description of the three neo-epitope antibodies used in this study, including the peptides used to generate the antibodies (caspase recognition sequence in bold). (D) By immunoblotting, the neo-htt586 antibody detects the truncated 1-586 amino acid fragments (15 or 128 CAG) but not huntingtin truncated at amino acid 552. As a control, an N-terminal huntingtin antibody (BKP1, right) was used to verify protein expression in extracts from these transfected 293 cells. (E) Immunofluorescent staining of COS cells transfected with cDNA encoding truncated huntingtin (1–586 amino acids or 1–552 amino acids) demonstrates that neo-htt586 specifically detects huntingtin when cleaved at amino acid 586. Transfected cells are indicated by huntingtin stain (mAb2166, red). Nuclei of cells are indicated by DAPI stain. (63× magnification, scale bar is 20 μm).
membrane. Very little staining was observed with the neo-htt513 antibody in untreated ST14 cells. A similar pattern of staining was seen with the neo-epitope antibodies for endogenously cleaved fragments in primary striatal neurons (Fig. 2B) and COS cells (data not shown).

Caspase-cleaved 586 amino acid fragments are rapidly processed

The low level of 586aa fragments generated by endogenous cleavage is difficult to detect by immunoblotting in whole
Figure 3. Endogenous huntingtin 586 amino acid fragments are increased by caspase inhibition and found in the nucleus. (A) Endogenously generated 1–586 amino acid fragments accumulate when further caspase proteolysis of huntingtin is blocked, suggesting 586 amino acid fragments are rapidly processed to smaller fragments. COS cells were transfected with 1–1212 amino acid huntingtin and left untreated (lane 2) or treated with a caspase inhibitor (5 μM zVAD for 1 h, lane 3). zVAD preferentially inhibits caspase-3 activity, and resulted in the accumulation of 586 amino acid fragments that can be detected with the neo-htt586 or huntingtin (BKPI) antibodies (red arrows). Similarly, point mutations that block caspase proteolysis of huntingtin at all caspase sites except amino acid 586 (1–1212 amino acid–4C, lane 4) dramatically increased the accumulation of the endogenously generated 586 amino acid fragments. Representative immunoblot is shown (n = 3 experiments), β-tubulin is the protein loading control. 586 amino acid fragments are not observed under the same conditions with the 5C construct (data not shown). Cells transfected with 1–1212 amino acid and then treated with exogenous purified C6 (lane 5) were used as a positive control for the 586 amino acid fragment. pCIneo control cells were transfected with the empty vector (lane 1). (B) Endogenously generated 586 amino acid fragment is enriched in the nuclear fraction. COS cells were transfected as indicated and lysates were separated into nuclear (N) and cytoplasmic (C) fractions. pCIneo (lane 1) is empty vector control. Endogenously generated 586 amino acid fragments (red arrows), which are enriched by blocking cleavage at other caspase sites in cells expressing huntingtin 1–1212 amino acid–4C (4C), were highly enriched in the nuclear fraction (lane 5). Lower panel is control immunoblot for the N/C separation procedure using antibodies against nuclear (PARP) and cytoplasmic (β-tubulin) proteins. (C) Schematic of the 4C and 5C constructs.

We hypothesized that the endogenously-generated 586aa fragments were rapidly processed by further proteolysis into smaller fragments by other caspases. To test this hypothesis, we used chemical inhibitors of caspases or genetic modification of caspase-cleavage sites to inhibit subsequent proteolysis to determine if 586aa fragments would accumulate and allow detection by immunoblotting (Fig. 3A).

Chemical inhibition of caspases was performed with zVAD, a broad spectrum caspase inhibitor. However, previous studies have shown that the affinity of zVAD is more than 2-fold lower for caspase-6 than caspase-3 (40). We hypothesized that zVAD would allow the accumulation of 586 amino acid fragments not by inhibiting caspase-6, but by inhibiting the further processing of 586 amino acid fragments by other caspases (such as C3). COS cells were transfected with DNA encoding 1–1212 amino acid huntingtin and treated with zVAD for 1 h. Compared with untreated cells, inhibition by zVAD increased the amount of 586 amino acid fragment detected by immunoblotting with both neo-htt586 and huntingtin (BKPI) antibodies. A C6 cut control (sample treated with recombinant C6 ex vivo) was used to clearly identify the molecular weight of the 586 amino acid fragments (Fig. 3A).

The crucial P1 aspartate at each cleavage site to an alanine, as described previously (4). Relative to unmodified huntingtin, transfection of the 4C construct results in a significant increase in the accumulation of 586 amino acid fragments in transfected but untreated COS cells (Fig. 3A, lane 4).

The 5C construct is a control construct in which all known caspase sites are blocked (amino acids 513, 530, 552, 586 and 589) and therefore cannot be cleaved at any of the known sites, including amino acid 586. As expected, transfection of the 5C construct did not result in the formation of 586 amino acid fragments (data not shown).

The chemical inhibition of caspases and genetic modification of caspase sites both increase the amount of endogenously generated 586 amino acid fragments in the absence of apoptotic stimuli. This is consistent with the hypothesis that there is a basal level of endogenously generated 586 amino acid that is rapidly processed by caspases to smaller fragments in vitro.

Enriched nuclear localization of caspase-cleaved huntingtin amino acid 586 fragments

To confirm that endogenously generated 586 amino acid fragments are specifically associated with the nucleus, we isolated the cytoplasmic and nuclear compartments from cultured cells for immunoblotting (Fig. 3B). COS cells were transfected with pCIneo-control, 1–1212 amino acid or 1–1212 amino acid–4C. As described (Fig. 3A), transfection of 1212 amino acid huntingtin did not result in detectable 586 amino acid fragments.
acid fragments in these cells. However, the 4C construct allows sufficient 586 amino acid fragment to accumulate for detection. Neo-htt586 and huntingtin (BKP1) antibodies both specifically detect the endogenously generated 586 amino acid fragments in the nuclear fraction relative to the cytoplasmic fraction (Fig. 3B, lane 5). Control proteins were immunoblotted to demonstrate that nuclear and cytoplasmic compartments were successfully isolated. As expected, PARP staining was enriched in the nuclear fraction while β-tubulin staining was almost exclusively cytoplasmic (Fig. 3B, lower panel).

Staurosporine induces caspase-6 activation and cell death

In order to develop a model to further understand the role of C6 and the generation of the 586 amino acid fragment, we chose to stress cells with staurosporine, as it is a general protein kinase inhibitor that has been used previously to induce cell death and activate C6. Visual assessment of untransfected ST14 cells demonstrates that 24 h of staurosporine treatment decreased the total number of cells and increased the number of cells with apoptotic morphology in a dose-dependent fashion (Fig. 4A). Cell viability was also reduced by staurosporine treatment in COS cells transfected with cDNA encoding 1–1212 amino acid–128Q huntingtin cells in a similar fashion. Staurosporine treatment resulted in a reduction in cell viability, as indicated by a dose-dependent decrease in ATP concentration (Fig. 4B) and an increase in C6 activation (Fig. 4C).

Endogenous caspase-6 zymogen is cytoplasmic and nuclear

Given the extensive nuclear localization of the endogenously generated 586 amino acid huntingtin fragments, we next determined the subcellular localization of C6. Punctate staining with the C6 (pan-C6) antibody, which detects both the pro- and active forms of C6, was found both in the nucleus and cytoplasm of untreated COS cells (Fig. 5A), ST14 cells and primary striatal neurons (Supplementary Material, Fig. S3). Staurosporine treatment was used to activate C6 and resulted in an increase in the nuclear staining with pan-C6, and was specifically associated with increased staining intensity at perinuclear microtubule-like structures (Fig. 5A). Representative images were taken with controlled exposure settings for comparison.

The perinuclear staining of C6 distinctly co-localized with γ-tubulin (Fig. 5B), a marker for the microtubule organizing center (MTOC). The intensity of pan-C6 staining associated with the MTOC and microtubule-like structures leading to the MTOC was increased following staurosporine treatment (Fig. 5B), and is consistent with the hypothesis that C6 is translocated to the MTOC and the nucleus following cell stress.
To determine whether the nuclear translocation of C6 was dependent on microtubules, cells were treated with nocodazole to disrupt microtubule networks and co-stained with pan-C6 and β-tubulin. In the absence of nocodazole, staurosporine treatment results in the translocation of pan-C6 to the MTOC and the nucleus (Fig. 6A). In the presence of nocodazole, however, staurosporine treatment was no longer able to stimulate this translocation (Fig. 6B, Supplementary Material, Fig. S4). This suggests that activation of C6 involves its translocation to the MTOC and nucleus, and this process is dependent on an intact microtubule network.

Active caspase-6 is nuclear

In contrast to the pan-C6 staining that was found throughout the cell, staining with an active-specific C6 antibody (cs-9761) demonstrates that active C6 is specifically nuclear (Fig. 7). Active-C6 staining was negligible in unstimulated cells. However, staurosporine treatment significantly increased the intensity of the active-C6 staining in the nucleus of ST14 (Fig. 7A), COS and primary striatal neurons (Supplementary Material, Fig. S3).

To determine whether the activation of C6 specifically occurred in the nucleus, cells were treated with nocodazole to disrupt microtubules, stimulated with staurosporine and stained for active-C6. In the presence of nocodazole, the intensity of active-C6 staining in the nucleus was significantly reduced (Fig. 7B, Supplementary Material, Fig. S4). Relative to cells with intact microtubule networks, nocodazole treatment reduced the intensity and frequency of active-C6 staining, suggesting that translocation of C6 is important to its activation. However, not all active-C6 staining was inhibited by nocodazole, suggesting that some pro-C6 is found in the nucleus and does not require translocation for activation. This is consistent with the pan-C6 staining, some of which was observed in the nucleus of unstimulated cells (Fig. 5).

Endogenously generated 586 amino acid fragments and active-c6 co-localize in the nucleus

Cell lysates were immunoblotted with the neo- htt586 antibody to determine whether staurosporine treatment, which results in the translocation and activation of C6 in the nucleus, also increased the amount huntingtin 586 amino acid fragment. As described (Fig. 3A), endogenously generated 586 amino acid fragments in whole cell lysates can be observed by immunoblotting when cells are transfected with the 4C construct or following zVAD treatment. Staurosporine treatment of these cells increased the intensity of the 586 amino acid band detected by immunoblotting (Fig. 8A, lanes 5 and 7). The intensity of the band was increased ~2-fold in both zVAD-treated and 4C construct-expressing cells (Fig. 8B).

The increase in 586 amino acid fragments was observed specifically in the nucleus. Immunofluorescent images taken with identical exposure settings demonstrate the increased intensity of neo-htt586 staining in the nucleus following staurosporine treatment (Fig. 8C). As expected, active-C6 and neo-htt586 staining co-localized (Fig. 8D), and is consistent with the proteolysis of huntingtin at amino acid 586 by C6 occurring in the nucleus.

Discrete pre-truncated caspase fragments have a different cellular itinerary than endogenously generated caspase fragments in cells

To determine whether discrete pre-truncated caspase fragments would traffic in the same manner as endogenously generated fragments, cells were transfected with cDNA constructs encoding huntingtin 1–513 amino acid, 1–552 amino acid or 1–586 amino acid. Unexpectedly, the discrete pre-truncated caspase fragments accumulated diffusely in the cytoplasm, regardless of fragment size. Similar results were observed in transfected ST14 cells (Fig. 9A) and COS (data not shown). The cytoplasmic accumulation did not vary with transfection expression level, as it was not altered between high or low expressing cells (data not shown).

To confirm the cytoplasmic distribution of pre-truncated caspase fragments, COS cells were transfected with cDNA encoding huntingtin 1–586 amino acid and lysates were separated into nuclear and cytoplasmic fractions. In agreement with the immunofluorescence data, the pre-truncated 1–586 amino acid fragments were enriched in the cytoplasmic isolate (Fig. 9A). PARP and β-tubulin controls confirmed the nuclear/cytoplasmic separation. The cytoplasmic accumulation of pre-truncated 1–586 amino acid fragments (Fig. 9B) contrasts sharply to the nuclear enrichment of endogenously generated 586 amino acid fragments (Fig. 3B). This data suggests that huntingtin sequences C-terminal to amino acid...
586, or post-translational modifications that result because of these sequences, are important for the specific localization of endogenously generated caspase fragments of huntingtin.

**DISCUSSION**

We have determined that endogenous caspase cleavage of huntingtin at different recognition sequences yields fragments with unique cellular distributions. Importantly, the endogenously generated 586 amino acid fragments are highly enriched in the nucleus. Caspase-6, when activated, is also enriched in the nucleus where it co-localizes with huntingtin 586 amino acid fragments. Thus, the nucleus is likely to be an important subcellular site of huntingtin amino acid 586 proteolysis.

Interestingly, neo-epitope staining for the endogenous caspase-2/3 552 amino acid fragments were enriched at the perinuclear region. This is consistent with previous reports of 552 amino acid fragments preferentially associating with membranes (5) at perinuclear sites (27). Although we could specifically detect over-expressed truncated 513 amino acid fragments with similar sensitivity to the other neo-epitope antibodies, we and others (27) have not detected endogenously generated fragments cleaved at amino acid 513. Rather, the lack of endogenous staining with the neo-htt513 antibody could be due to either a low abundance of endogenously generated 513 amino acid fragments or masking of the C-terminal epitope. The specific and different subcellular localization of endogenously generated 586 amino acid and 552 amino acid fragments suggest that these fragments are either trafficked to these precise locations after cleavage or that huntingtin cleavage only occurs at these specific locations in the cell.

Consistent with previous reports (30,34), active C6 immunostaining was concentrated in the nucleus and coincided with the nuclear localization of 586 amino acid fragments of huntingtin. Immunostaining for pan-C6 revealed a punctate pattern in both the cytoplasm and the nucleus of ST14 cells and primary striatal neurons. Stimulation of these cells with staurosporine, a kinase inhibitor known to activate cell death signalling pathways, resulted in a dramatic enrichment of nuclear C6, both with the pan-C6 and active-C6 antibodies. Interestingly, staurosporine also induced the translocation of C6 from the cytoplasm to the nucleus. This translocation is dependent on microtubule networks as it could be eliminated by a microtubule-disrupting agent, nocodazole. Pan-C6, but not active-C6, co-stains with γ-tubulin at the MTOC.
tin is also found at the MTOC and is known to interact with microtubules (41,42). Many C6 substrates, such as Lamin A and PARP, are nuclear or predominantly cleaved in the nucleus (Supplementary Material, Table S1). Our data is most consistent with the C6 cleavage of huntingtin at amino acid 586 taking place in the nucleus.

Although there may be other, yet unknown proteases capable of cleaving htt at amino acid 586, numerous lines of evidence suggest that C6 is the major protease cleaving htt at this site: Purified caspase-6 specifically generates a 1–586 amino acid fragment that is detected by htt antibodies (4,14) and the cleavage-specific neo htt586 antibody (Fig. 3, 8 and 9). Often caspases can be promiscuous in terms of sharing substrate cleavage sites. However, we have assessed and not found another purified protease (caspase 1–10 or any calpain) that can generate the 1–586 amino acid fragment. In addition, IVLD is a unique cleavage site for caspase-6, and htt is the only substrate with this recognition site identified thus far (Supplementary Material, Table S1). Furthermore, active caspase-6 is elevated in HD (29). Additional evidence

Figure 7. Active capase-6 staining is nuclear. (A) Minimal active-C6 staining (cs-9761) is observed in untreated ST14 cells. However, following staurosporine treatment (0.2 μM, 1 h), active-C6 staining is increased specifically in the nucleus. Exposure settings were held constant in the representative images to allow comparison. (B) Nuclear activation of C6 is partially inhibited by nocodazole-induced disruption of microtubule networks. Nocodazole treatment (10 μM, 1 h) breaks up microtubule networks, as indicated by the diffuse β-tubulin staining (red) and results in a reduction in the intensity of the nuclear active-C6 staining. This data is consistent with the hypothesis that a portion of cellular C6 is translocated to the nucleus on microtubules before being activated. 100× magnification (1,40 Oil), scale bar is 20 μM.
is provided by the finding that active C6 and htt-586 amino acid co-localize in the nucleus (Fig. 8).

The order of huntingtin cleavage events that occur under normal physiological conditions is not well understood. At least two models are feasible: the shorter amino acid 552 fragment could be generated from the longer amino acid 586 fragment and exported from the nucleus, or alternately, amino acids 552 and 586 cleavage could represent parallel, rather than sequential, cleavage events (Fig. 10). Our data with zVAD and 4C cleavage-mutant constructs are most compatible with the former hypothesis and suggest that under normal physiological conditions there is a low basal level of endogenous amino acid 586 fragments that are turned over rapidly by processing to smaller fragments and translocated out of the nucleus.

Figure 8. Staurosporine increases endogenously generated 586 amino acid fragments in the nucleus. (A) Staurosporine increased the intensity of the 586 amino acid band detected by immunoblotting in COS cells transfected with 1–1212 amino acids or 1212 amino acid+4C huntingtin. 1–1212 amino acid huntingtin-expressing cells were treated with zVAD (lane 4) or zVAD+staurosporine (lane 5). 1–1212 amino acid+4C huntingtin-expressing cells were untreated (lane 6) or treated with staurosporine (lane 7). (B) The intensity of the 586 amino acid band is quantified and is approximately increased 2-fold in both 1–1212 amino acid+zVAD or 1–1212 amino acid+4C cells (n = 3). (C) Staurosporine treatment increases the intensity of nuclear neo-htt586 staining in ST14 cells. Representative pictures from cells +/- staurosporine treatment taken at the same exposure settings. (D) Endogenously generated 586 amino acid fragments and active C6 co-localize in the nucleus. Untransfected ST14 cells were stressed with staurosporine (100 μM, 1 h) and stained with the neo-htt586 and C6 active polyclonal (cs9761). 100 x magnification (1.40 Oil), scale bar is 20 μM.
Relative to other caspase fragments, the unique nuclear enrichment of 586 amino acid fragments may explain why this specific fragment is important in the pathogenesis of HD. In prior studies, blocking cleavage at amino acid 586 prevented the development of motor, behavioural and neuropathological features in the C6R-YAC128 mice (14). Blocking cleavage at other caspase sites, including amino acid 552 in the C3R-YAC128 did not result in protection from the toxic effects of full-length polyglutamine-expanded huntingtin. The current data suggest that the specific nuclear accumulation of amino acid 586 fragments may distinguish them from other fragments that are less toxic.

Numerous studies have suggested that a portion of cellular huntingtin, including full-length huntingtin, is found in the nucleus (27,28,43–46) and are consistent with nuclear transport of huntingtin as part of its normal itinerary (47–50).

The HEAT repeat and PEST predictions of huntingtin structure suggest that proteolytic processing of huntingtin is important in the function of the protein, rather than simply a pathway for degradation and removal. Full-length huntingtin is almost entirely composed of HEAT repeats (35–37), which may play a role in the regulation of its nuclear shuttling. HEAT repeats regulate protein–protein interaction and are frequently involved in nuclear transport (35). The location of the PEST sequences, regions of proteolytic susceptibility, strongly suggest that huntingtin proteolysis serves a specific function—to disrupt or separate modular HEAT domains and the proteins bound by them. Interestingly, some PEST sequences in other proteins are conditionally active and require post-translational modifications such as phosphorylation to regulate proteolysis (38).

Examination of the subcellular localization of huntingtin caspase fragments reveals important differences in the cellular itinerary of endogenously cleaved compared with discrete pre-truncated caspase fragments. We found that pre-truncated fragments (amino acids 1–586, 1–552 or 1–513) all similarly accumulated in the cytoplasm regardless of the absolute level of expression or the size of the fragment. The first 17 amino acids of huntingtin function as a cytoplasmic retention signal (50,51) which is likely a crucial factor in determining the localization of shorter huntingtin fragments. This is in contrast to the specific localizations of the endogenously generated 586 and 552 amino acid fragments originating from longer forms of huntingtin.

One important limitation of many of the current truncated HD mouse models (26,52,53) is that the truncation has been selected based on genetic (i.e. protein fragment contained in a particular subset of exons, convenient restriction sites for DNA cloning, random gene truncation) rather than molecular or biochemical criteria (i.e. authentic cleavage sites, HEAT domains). The clear difference between the trafficking and localization of the different fragments, particularly between pre-truncated fragments and endogenously cleaved fragments, emphasizes the importance of the size of the fragment, as well as how and where the fragment is generated. This finding has important implications for the interpretation of models of HD using truncated fragments of huntingtin. Truncated in vitro and in vivo models of HD may not be modeling the specific pathological processes of the disease because these ‘pre-cleaved’ truncated fragments may not enter the correct cellular pathways. The shortstop mouse, for example, expresses exons 1 and 2 of the HD gene and has extensive inclusions, but unexpectedly, does not have a neuropathological phenotype (26).
Numerous studies have emphasized the importance of nuclear huntingtin in the development of HD (12,24,54–56). Indeed, the nuclear localization of another polyglutamine-expanded protein, ataxin-1, has been shown to be crucial to the development of the disease phenotype models of SCA1 (57,58). Previous hypotheses of HD pathogenesis have focused on proteolysis strictly as a means of degradation of huntingtin and have involved the proteolytic cleavage of huntingtin in the cytoplasm (Fig. 10A), allowing the translocation and cytotoxic accumulation of polyglutamine-expanded fragments (59–62).

The current findings, however, are consistent with the hypothesis that full-length huntingtin is trafficked to the nucleus where it is cleaved by C6 (Fig. 10B). Both 586 amino acid fragments and active-C6 are concentrated in the nucleus. These data suggest a basal level of endogenously generated 586 amino acid fragments can be increased by cellular stress, which also causes the nuclear translocation and activation of C6. Therapeutic strategies that regulate the nuclear translocation of huntingtin or C6, and/or specifically enhance the nuclear export of 586 amino acid fragments, may be a viable means of altering this important proteolytic event.

**MATERIALS AND METHODS**

**Huntingtin structural predictions**

The location of HEAT repeats was predicted using the REP_tool (http://www.embl-heidelberg.de/~andrade/papers/rep/search.html) (63). Twenty-six HEAT repeats were detected and clustered into HEAT domains based on proximity to each other (Fig. 1A). The location of PEST sequences was determined using the PEST_find tool (http://bioweb.pasteur.fr/seqanal/interfaces/pestfind.html) (38). Six PEST sequences were clustered into four PEST domains based on proximity (Supplementary Material, Fig. S1).

**Tissue culture and DNA constructs**

Cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS + L-glutamine (Gibco). All lines were incubated at 37°C and 5% CO₂ except for ST14A cells, which proliferate at 33°C (64,65).

Cultured primary striatal neurons were dissected from 6–10 newborn FVB pups (P0-1) and cultured for 9–10 days (66) before fixing for immunofluorescence. Transient transfection of COS and 293 cells were performed with Fugene 6 (Roche) following the manufacturer’s standard instructions. Briefly, cells were transfected in 6 well (1 µg DNA + 3 µl Fugene reagent) or 10 cm dishes (8 µg DNA + 24 µl Fugene reagent) and then harvested 18 h later unless otherwise indicated. ST14A cells were transfected in 24-well dishes with Lipfectamine 2000 (Invitrogen) based on the manufacturer’s protocol, using 0.6 µg DNA and 3 µl Lipfectamine reagent per well. The DNA constructs used for transient transfection were in the pcineo mammalian expression vector and were either constitutively truncated caspase fragments (amino acids 1–513, 1–552, 1–586) or 1–1212 amino acid (3949nt–15Q or 3949nt–138Q). 1–1212 amino acid + 4C constructs were used to enhance the ability to detect the 1–586 amino acid fragment by blocking the subsequent processing of the protein at other caspase sites. 4C refers to the P1 aspartate-to-alanine point mutations at four predicted caspase sites (amino acids 513, 530, 552, 586 and 589). The 5C construct is a control construct in which all caspase sites are mutated and blocked (amino acids 513, 530, 552, 586 and 589) (4).

**Cell viability assays**

Cell viability following staurosporine (Sigma-S4400) treatment was assessed visually on a Zeiss Axiovert 25 with a 10× (0.25 PH1) objective. Images were captured on a Nikon D70 with a 0.25× converter. Culture plates were marked so that the same cells could be photographed before and after treatment.
Cellular ATP levels were determined by following the instructions in the Cell-titer Glo kit from Promega (Madison, WI, Cat. #G7573). Briefly, media was removed from the cells and replaced with 150 μl/well of cell-titer glo reagent mixed 1:1 with fresh DMEM. Cells were lysed on an orbital shaker at room temperature for 10 min, and 100 μl supernatant was transferred to black 96-well plates. Luminescence was integrated for 1 s per well on a Victor3 plate reader (Perkin Elmer, Waltham, MA), and values were normalized to untreated control wells on the same plate.

Caspase-6 activation was assessed with a fluorogenic VEID-assay. Whole cell extracts from staurosporine treated cells were incubated with 50 μM Ac-VEID-AFC (BioMol P439) in caspase assay buffer (50 mM Hepes pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT) at 37°C for 1 h. Cleavage of Ac-VEID-AFC to release AFC by active C6 was measured (405 nm excitation; 535 nm emission) in a Victor3 plate reader.

**Cell lysis**

Cultured cells were harvested by trypsinization. Lysis was performed in the presence of 1× Roche complete protease inhibitor, 1 mM sodium orthovanadate and 800 μM PMSF; 5 μM zVAD was included in nuclear and cytoplasmic extractions. Protein concentrations were determined by the Bradford Assay (BioRad).

Whole cell extracts were lysed in SDP+ buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Igepal, 40 mM B-glycerophosphate, 10 mM NaF, 1× Roche complete protease inhibitor, 1 mM sodium orthovanadate and 800 μM PMSF), incubated on ice for 15 min and sonicated briefly to shear DNA. Debris was removed by centrifugation (15 min, 20 000g, 4°C) and the supernatant retained. For C6-cut controls, samples were treated with purified C6 (BioMol SE-170, 30°C, 1 h).

Nuclear and cytoplasmic extracts were prepared by separating cell contents into a supernatant and pellet corresponding to cytoplasmic and nuclear compartments of the cell as previously described (67), noting the following modifications. The pellet containing the crude nuclear compartment was isolated by centrifugation (4 min, 8200 g, 4°C), the supernatant retained as the cytoplasmic compartment and then the nuclear pellet was washed with PBS and lysed in nuclear lysis buffer. Following the 30 min incubation on ice, an equal volume of 1% TritonX-100 in TEEN buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) was added to the nuclear extract, mixed by vortexing and left on ice for an additional 5 min. The nuclear lysate was then sonicated to shear DNA.

**Antibodies**

Immunoblots for huntingtin were conducted using mAb2166 (Chemicon monoclonal 4C8), BKPI (huntingtin N1–17 amino acid) and the neo-epitope antibodies. The neo-htt586 rabbit (polyclonal) and mouse (monoclonal) antibodies were generated from the 581IVLD586 epitope found at the C6 cleavage site in huntingtin. Neo-htt552 and neo-htt513 antibodies have been described previously (39). Other antibodies used include: pan-C6 (Santa Cruz-15381), active-C6 (Cell Signalling-9761), γ-tubulin (Abcam-11316), β-tubulin (Sigma-4026) and PARP (Cell Signalling-9542).

**Immunoblotting**

Standard procedures were used for immunoblotting. Briefly, sample preparation involved denaturing the lysates or immunoprecipitates in LDS Sample Buffer (Invitrogen) or SDS gel loading buffer with heating for 10 min at 70°C. For huntingtin immunoblots, a 3–8% Tris–acetate gel (Invitrogen) was run with Tris–acetate buffer (50 mM Tricine, 50 mM Tris-base, 3.5 mM SDS, pH 8.25) for 1 h at 200 V. Transfers were performed onto Immobilon-PVDF-FL membranes at 30 V for 1.5 h using transfer buffer (25 mM Bicine, 25 mM Bic–Tris, 1.025 mM EDTA, 10% MeOH, pH 7.2). Blocking was performed with 5% milk in PBS or TBS depending on the primary antibody.

All immunoblots used IR dye 800CW goat anti-mouse (Rockland 610-131-007) and AlexaFluor 680 goat anti-rabbit (Molecular Probes A21076) labeled secondaries, and the LiCor Odyssey Infrared Imaging system. For statistical analysis, groups were compared based on the numerical densitometry results from Licor Odyssey software (v2.0). A t-test was performed with two groups and one-way ANOVA was performed for more than two groups. P-values <0.05 were considered to be statistically significant.

**Immunofluorescent microscopy**

Immunofluorescence was performed in cultured cells grown on coverslips using standard procedures. Following staurosporine treatment (0.2 μM, 1 h), cells were fixed for 2 min with ice-cold MeOH, permeabilized in 0.3% Triton X-100 in PBS for 30 min and washed with PBS. Cells were then blocked in 4% normal goat serum (Gibco) in PBS for 1 h at room temperature, incubated with the primary antibodies in 2% normal goat serum in PBS for 2 h at room temperature, washed 3× with 1% BSA in PBS and then incubated in the secondary antibodies (Goat anti Mouse Alexa 568 (red); 1/800 or Goat anti Rabbit Alexa 488 (green); 1/800) in 2% normal goat serum in PBS for 2 h at room temperature. Cells were washed in PBS before mounting; DAPI staining (1:10 000 in PBS from 10 mg/ml stock in 70% ethanol, SIGMA) was included in the first wash (room temperature, 5 min). Fluoromount-G (Southern Biotech Cat. #0100–01) was used to mount coverslips on slides. Immunofluorescence was detected using a laser confocal microscope (BioRad) or conventional immunofluorescence microscopy (Zeiss Axioplan2) with a CCD camera (Princeton Instruments Inc.); images were captured using Metamorph software (version 6.1r3). Images within a figure panel were taken with the exposure settings held constant for each channel and are representative of experiments and staining performed at least three times.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.
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