

# **The Role of Proteolysis in the Pathogenesis of Huntington's Disease**

December 4-5, 2001  
Buck Institute for Age Research  
Novato, California

Prepared by Marina Chicurel, Ph.D.

## **Abstract**

Despite a growing number of clues, the molecular mechanisms underlying Huntington's disease (HD) remain elusive. One of the leading proposals, the toxic peptide hypothesis, postulates that the cleavage and accumulation of fragments of huntingtin protein, bearing an expanded polyglutamine region, lie at the heart of HD's pathology. The hypothesis is far from proven, however. Participants at the workshop "The Role of Proteolysis in the Pathogenesis of Huntington's Disease" discussed the accumulated evidence in favor and against the proposal, and identified experimental approaches to resolve some of the open questions and accelerate the search for therapeutic candidates.

Participants agreed that the identification and characterization of proteolytic huntingtin fragments is a priority, and suggested extending and improving antibody-based studies, as well as performing mass spectrometry and X-ray crystallography to characterize the fragments' sequence, secondary modifications, and structure. They also discussed using genetic-based assays and protease inhibitors to identify the relevant proteases. Several approaches, including the use of 'suicide substrates,' fluorescent emitters and quenchers, and expression microarrays, were suggested to track protease expression and activity. Identifying the order of cleavage and determining the relevance of fragment subcellular localization were underscored as important goals. Ideas for initial steps to search for therapeutic candidates included performing cell-based screens for inhibitors of huntingtin cleavage, and assessing the effects of upregulators of proteasome function and the heat shock response.

## **Powerful motivators for unraveling HD's mechanisms**

The experiences described by a couple whose family is afflicted with HD and a heart-wrenching videotape of HD victims in a Venezuelan community, fuelled participants' desire to develop strategies to understand the mechanisms underlying HD.

Underscoring the relentless progression of HD, the invited couple recounted how their 44 year-old son has been transformed by the disease since he first developed symptoms more than 10 years ago. They described their son as having been an athletic young man who excelled in school and at his job, only to begin experiencing signs of poor judgement, anxiety, and depression, which later gave way to the more typical symptoms of HD –pronounced cognitive deficits and impaired motor skills. Today, he lives with his parents, unable to balance his checkbook, or engage in any of the sports he once enjoyed.

Guided by Nancy Wexler's explanations, participants also watched video clips shot in Venezuela showing the slow movements of youngsters suffering from juvenile HD, the choreic movements and difficulties walking of people afflicted with adult-onset HD, and the terminal stages of the disease, characterized by severe undernourishment, and the loss of virtually all motor abilities. The scenes were particularly harrowing since the patients live in abject poverty and HD is widespread in their highly inbred community.

## **The toxic peptide hypothesis: many unanswered questions**

Hoping to contribute to unraveling the mechanisms underlying such a devastating disease, participants discussed the toxic peptide hypothesis –a proposal suggesting that the main culprit of HD pathology is the cleavage and build-up of mutant huntingtin fragments. As noted by Allan Tobin, the origins of the hypothesis date back to 1996, when Gillian Bates and her colleagues showed that transgenic mice carrying only a fragment of the huntingtin gene--exon 1

with an expanded CAG repeat-- suffer from a progressive neurological phenotype similar to human HD. Shortly after, in 1997, Marian DiFiglia and colleagues found evidence of huntingtin processing in HD, showing that neuronal inclusions present in HD brains could be immunolabeled with antibodies directed against the N-terminus, but not the C-terminus, of huntingtin protein. Since then, numerous reports have suggested that mutant huntingtin's toxicity arises from its cleavage and the accumulation of amino-terminal fragments within specific neuronal populations.

As described by Blair Leavitt, testing the toxic peptide hypothesis requires two basic approaches: designing experiments to re-create toxicity in model systems using huntingtin fragments, and assessing whether cleavage occurs at the right time and the right place during disease progression. Several reports have confirmed that N-terminal fragments of mutated huntingtin, and more specifically, as noted by DiFiglia, fragments spanning the first 500 amino acids of the protein, are indeed toxic. Furthermore, these fragments are usually more toxic than the full-length protein. Studies have also shown that toxicity can be reduced by inhibiting caspases, or mutating huntingtin's caspase cleavage sites. In support of cleavage occurring in vivo, N-terminal fragments presumably generated by caspases and calpain have been found in HD brains, as well as in animal and cell culture models of disease.

However, the toxic peptide hypothesis is far from proven. It remains uncertain whether huntingtin fragments are generated as part of the disease process, and if so, whether they act as specific causal agents of disease, disrupt the cells' turnover machinery non-specifically, or are byproducts of other primary pathological events. Neither the identity of the huntingtin fragments, nor the proteases that generate them have been unequivocally established. The differences in processing between mutant and wildtype huntingtin have yet to be resolved, with some experiments suggesting that, in fact, few differences exist.

In addition, some studies suggest alternatives that are altogether incompatible with the hypothesis. Cynthia McMurray, for example, noted that her results indicate mutant huntingtin in human brains remains, for the most part, intact. In a recent article, she reported that mutant huntingtin is more resistant to proteolysis than the normal protein, and suggest a model in which *inhibition* of proteolysis of mutant huntingtin causes aggregates to form, which cause damage by sequestering other cellular proteins, including normal huntingtin fragments. Christopher Ross and Leavitt cautioned, however, that McMurray's absence of evidence is not necessarily evidence of absence. Hook added that it is difficult to conclude at this time that mutant huntingtin is more resistant to proteolysis than the normal protein because some studies have been conducted under native conditions, while others have used denaturing procedures. Another problem, noted Xiao-Jiang Li, is that the huntingtin fragments may be unstable and consequently difficult to detect under certain circumstances.

Part of the reason for the large number of uncertainties in the field is the variation in experimental techniques and model systems. McMurray noted, for example, that animal models that develop advanced symptoms at early ages, such as R6/2 mice, may suffer from a different pathology than models which follow a slower progression, such as knock-in mice. In her opinion, the accelerated models are probably closer to re-creating juvenile HD, whereas the slower models, which generate fewer huntingtin fragments, more closely mirror adult-onset HD.

As described by Vivian Hook, variations in the conditions used for Western blots can also greatly affect an experiment's results. Parameters such as the choice of buffers, presence of protease inhibitors, denaturing conditions, and blocking protocols are far from standardized and are likely to underlie some of the discrepancies observed between studies. Louis Hersh

emphasized denaturation as a particularly important source of variability due to its influence on antibody binding. Although proteins are denatured during SDS-PAGE, some degree of renaturation can occur during transfer.

Niamh Cawley noted that the ability of N-terminal fragments to re-assemble into dimers or higher order oligomers during electrophoresis may be a particularly important source of altered mobility and variability, especially in the case of material derived from inclusions which are initially resistant to SDS solubilization. During electrophoresis and in the absence of significant heat, re-folding and re-formation of aggregates or oligomers may occur even in the presence of SDS. In addition, Cawley pointed out that full length huntingtin is usually assumed to be the slowest migrating immunopositive band on Western blots. Yet on small gels, the accuracy is generally within 10%, yielding a potential variation of at least 35kDa for a 350kDa protein. If processing were occurring at either end of the protein, the remaining fragment may not show the shift.

Differences in the choice of antibodies have also made comparisons between studies difficult (Table 1). McMurray, for example, noted that several studies have relied on antibodies that bind to the N-terminal of huntingtin, but do not distinguish between mutant and wildtype proteins, making it difficult to discern the origins of fragments. Hook commented that the use of samples that are homozygous for mutant huntingtin can allow direct investigation of the mutant, rather than normal, protein.

Differences in the behavior of huntingtin within distinct subcellular locations add yet another layer of complexity. Li and Yvon Trottier reported subcellular-specific immunostaining of huntingtin fragments. One possibility is that the observations reflect differences in the presence of huntingtin fragments. But another possibility, suggested by Tobin, is that the fragments' epitopes may be differentially accessible. In particular, the degree of a fragment's association with aggregates may be particularly important in determining its immunoreactivity. Aggregates may mask epitopes, making fragments less detectable, or alternatively, they may stabilize fragments, making them more detectable. Also, Cawley noted that studies relying on immunoreactivity to compare the abundance of huntingtin fragments in normal and diseased tissues may be misleading since epitope masking appears to vary between the normal and mutated protein. Indeed, some antibodies preferentially bind the mutant protein, even though they appear to recognize non-poly-glutamine regions whose sequence is identical in both proteins (Table 1).

### **Identifying the relevant fragments**

Several participants agreed that a first step towards untangling the uncertainties in the field is to characterize the huntingtin fragments generated during the course of HD. Recently identified fragments --including ones described by Trottier, Li, Hook, Akira Sawa, Anja Dröge, and DiFiglia-- were fingered as important targets for further characterization.

Of particular interest was a small fragment found in nuclear inclusions by Trottier. Using a new panel of antibodies to investigate the immunogenic properties of cytoplasmic and nuclear aggregates in HD brains, Trottier and his colleagues found that cytoplasmic and nuclear aggregates appear to be composed of huntingtin fragments of different sizes. In particular, they observed that in NG108 cells expressing either full-length or a 500-amino acid N-terminal fragment of mutant huntingtin, antibody 1H6 immunoreacted with some cytoplasmic, but not nuclear, aggregates. Since the epitope of 1H6 maps to amino acids 115-125, the researchers reasoned that a major component of nuclear aggregates might be huntingtin fragments spanning

less than 115 amino acids. Evidence for short fragments in the nucleus was also presented by Li, who was unable to stain nuclear inclusions using the recently developed EM121, a rabbit polyclonal antibody generated using huntingtin amino acids 342-456.

Confirming that this inability to detect regions of huntingtin beyond amino acid 115 is due to the presence of short fragments --rather than to the selective masking of epitopes in the nucleus-- Trottier's team identified a small fragment on Western blots as the major component of nuclear inclusions. Using a disaggregation protocol that relies on treatment with formic acid, the researchers identified a fragment migrating at 55kDa. Comparing this migration with that of genetically engineered fragments of known size, they found that the fragment is indeed small, as suggested by the antibody data. Taken together with Trottier's observations indicating caspase cleavage occurs after inclusion formation, these data suggest that the generation of the 55kDa fragment might be a primary event in the formation of inclusions.

### ***Tapping into antibodies' full potential***

To further characterize such fragments and help researchers compare their results, Hook and Cawley stressed the importance of standardizing gel electrophoresis and Western conditions. In particular, Cawley recommended adopting conditions that strip away higher-order structure as much as possible.

In addition, participants suggested characterizing available antibodies more thoroughly, performing epitope mapping for those whose recognition sites remain unknown. In particular, participants proposed mapping EM 48, an antibody developed by Li described as the best available for detecting fragments with poly-glutamine expansions. EM 48 was generated using a GST-fusion protein carrying the first 256 amino acids of huntingtin as an immunogen. Surprisingly, however, the antigen completely lacked both poly-proline and poly-glutamine stretches. As noted by Ross, who has produced similar antibodies, this discrepancy between the immunogen and the antibody's affinity suggests that fragments with expanded repeats may expose certain epitopes better than fragments with normal repeats. This may occur, as suggested by Cawley, by either conformational re-folding of single molecules, or by oligomerization. Thus, mapping EM 48's epitope promises to yield clues about how expansions affect huntingtin's conformation or aggregation properties.

Despite the numerous antibodies already available (Table 1), participants agreed that additional ones would be valuable. Lisa Ellerby, for example, has recently generated antibodies that recognize huntingtin regions that include caspase cleavage sites, and is now developing ones that recognize calpain cleavage sites.

Participants also suggested using antibodies to perform *in vitro* experiments to examine huntingtin processing. For example, Li described experiments in which he incubated an *in vitro* synthesized fragment of huntingtin, consisting of the protein's first 500 amino acids, with brain extracts from mice. His preliminary data suggest brain proteases can generate several huntingtin fragments, including one migrating at 45kDa which he observed even when he pre-treated extracts with caspase and proteasome inhibitors. Li is planning to test various protease inhibitors, as well as perform biochemical purifications and enrichments using high-performance liquid chromatography to identify the protease or proteases involved. This assay may also help uncover cellular factors that influence the stability of huntingtin, he said, which can be tested further using *in vivo* models such as HD knock-in mice.

In addition, Cawley noted that *in vitro* systems might be useful for identifying differences in the proteolytic cleavage of wildtype and mutant huntingtin. Since processing may be only

slightly different between the two proteins, Cawley suggested testing several conditions. He noted that Michael Hayden's group has performed *in vitro* experiments in which they observed cleavage when using apoptotic, but not control, cell extracts. However, Hayden did not use full-length huntingtin and his incubations were limited to an hour. It is possible, suggested Cawley, that low level processing occurred in the controls but was undetectable at that time point. Given the slow progression of the disease, time may be a critical factor. In addition, analysis of the full-length protein may yield new insights.

Jeffrey Stack cautioned, however, that *in vitro* experiments can generate artifactual fragments because the conditions do not match those existing within cells. In addition, performing experiments with full-length huntingtin may prove difficult because previous attempts to produce the protein using bacteria and baculovirus systems have failed.

### ***Fragment sequence and structure***

Many participants agreed that to characterize huntingtin fragments in detail, they would need proteomic techniques such as mass spectrometry. As noted by Bradford Gibson, SDS-PAGE is unreliable for sizing proteins and antibodies can be misleading. Using mass spectrometry, however, molecular weights can be rigorously determined, and fragments can be unequivocally identified by sequencing, without requiring large amounts of sample proteins. According to Gibson, mass spectrometry is reliable in the picomole to femtomole range and, in some cases, even down to attomoles. Sawa noted that this sensitivity is more than sufficient for analyzing huntingtin fragments recovered from lymphoblasts, and that he is in the process of collecting material for this purpose.

To assess the feasibility of using the technique to analyze HD inclusions, Tobin made a rough calculation of the amount of material one might be able to extract from an inclusion. The estimate suggested that analyzing inclusion composition may indeed be possible. In addition, as noted by Cawley, inclusions' unique resistance to SDS extraction could facilitate their purification.

Predicting the setbacks that may arise during mass spectrometry analysis, however, is difficult. Referring to his previous work with prion proteins, Gibson noted that much time must often be invested in optimizing detergents, salt concentrations, and the type of instrument used. On the other hand, sometimes anticipated difficulties are not encountered. For example, proteins that tend to aggregate may turn out to be easily manipulated in the gas phase.

In addition to its potential for revealing the identities of huntingtin fragments, Gibson emphasized that mass spectrometry can help identify post-translational modifications by allowing the detection of molecular weight differences as small as a single Dalton. One modification that might be particularly relevant to the behavior of huntingtin fragments is glutamine de-amidation. Noting that glutamine in solution is extremely unstable, Alfred Goldberg wondered about the amino acid's stability within the huntingtin protein. Although Trottier's mass spectrometry analyses of GST fusions of huntingtin fragments don't seem to indicate the presence of modifications, Goldberg pointed out that additional experiments are needed to assess whether the modifications accumulate over time. In addition, as explained by Gibson, small shifts in molecular weight can be easily missed.

Several participants also stressed the importance of obtaining structural information and comparing it between wildtype and mutant fragments. According to Carl Johnson, several labs are currently working to obtain crystal structures of huntingtin's exon 1. In addition, McMurray is collaborating with crystallographers to set up methods to purify and crystallize full-length

huntingtin. Other approaches included Gibson's proposal of using limited proteolysis *in vitro* to identify the proteins' exposed and hidden domains. Gibson also suggested using chemical cross-linkers to help assess the domains' relative positions.

### **Identifying the relevant proteases**

Caspases and calpains have been implicated in huntingtin cleavage, but their roles *in vivo* remain elusive. In addition, the identification of new fragments, such as Trottier's, suggests the involvement of additional proteases. Participants proposed using genetic-based assays, protease inhibitors, and labeling techniques to monitor proteases and pinpoint their roles in huntingtin processing.

### ***Genetic-based strategies***

Of particular interest were experiments described by Cheryl Wellington working in Hayden's lab that have the potential of directly linking specific cleavage sites to HD pathology. In a recent study, Wellington generated site-directed mutants that rendered huntingtin resistant to caspase-3 cleavage at positions 513 and 530, and to caspase-6 at position 586. The researchers observed that, compared to cleavable huntingtin, the mutant proteins were less toxic and formed aggregates less frequently when expressed in apoptotically stressed cells. Now the group is setting up similar experiments *in vivo* using the YAC mouse model of HD. The team is generating a set of mice bearing mutations in each of huntingtin's known caspase cleavage sites. Wellington estimates the ambitious project will be finished within a year and a half. It should then start generating data that, not only link specific cleavages to pathology and symptoms, but illuminate the sequence of events underlying proteolytic processing by revealing which cleavages are required for other cleavages to occur.

As noted by Hersh, however, performing a comprehensive evaluation of all protease sites represents a tremendous undertaking. In addition, the creation of some cleavage mutants may result in "cryptic" secondary protease sites that could cause artifactual processing and interfere with the interpretation of the results.

Another genetic strategy briefly discussed was the creation of dominant-negative protease mutants. Previous work by Robert Friedlander showed that expression of a dominant-negative caspase-1 mutant extends the survival of R6/2 mice and delays the appearance of neuronal inclusions, neurotransmitter receptor alterations, and the onset of symptoms. Although these effects are thought to be due to an inhibition of apoptosis, rather than huntingtin cleavage, additional dominant-negative mutants, such as those being generated by Ellerby, may shed light more directly on the effects of interfering with huntingtin processing. As noted by DiFiglia, however, redundancy in protease function could limit the power of this approach.

### ***Protease inhibitors***

Participants also pointed out that the full potential of protease inhibitors, both as tools to dissect the roles of proteases in huntingtin processing and as candidates for therapeutic intervention, has yet to be tapped. Goldberg noted that several companies have developed protease inhibitors that might be useful for studying huntingtin processing. For example, companies have made significant investments in developing inhibitors that can cross the blood brain barrier to treat diseases such as stroke. Parke-Davis and Alkermes have produced high quality calpain inhibitors and, as noted by Stack, Merck has developed small peptide inhibitors for caspases. Vertex, which recently merged with Aurora Biosciences, has a caspase-1 inhibitor,

although Stack noted it might be hard to obtain. There may also be valuable compounds sitting on companies' shelves. For example, as noted by Goldberg, a company searching for an inhibitor of inflammation that does not reach the brain, may set aside protease inhibitors that cross the blood brain barrier which could be useful for HD research.

The versatility of 'suicide' substrates described by Mathew Bogyo also generated much excitement. These inhibitors consist of a peptide bearing a tag, such as biotin, or a fluorescent or radioactive moiety, and an 'electrophyle,' such as an epoxide derivative. Tailored for specific enzymes, these substrates are recognized only by active enzymes, and bind irreversibly to their targets. 'Suicide' substrates can thus be used to selectively inhibit or label active enzymes. For example, they can be used to monitor enzyme activity in *in vitro* screens of small molecule inhibitors, to follow the time course of an enzyme's activity within a mouse tumor, or to knock out an enzyme in cell culture or in an adult animal. So far, Bogyo and his colleagues have generated 'suicide' substrates for calpains and lysosomal enzymes, and they are currently developing substrates for caspases and other enzymes. Thus, these reagents might help create a roadmap of protease activation in models of HD.

Although participants emphasized the need for specific protease inhibitors, they also noted the benefits of general protease inhibitors. Robin Thurmond, for example, opined that the information available on huntingtin processing was insufficient to initiate high throughput screens using specific inhibitors, and that broad-based inhibitors could be used as a first step to home in on specific targets. Bogyo suggested that the effects of general inhibitors could be relatively quickly and cheaply assessed using SDS-PAGE to monitor cleavage patterns of huntingtin in *in vitro* experiments using cell extracts. Also supporting the value of general inhibitors, Ellerby suggested performing experiments using cocktails to inhibit caspases and calpains simultaneously. Besides offering broad-based probes for studying huntingtin processing, general inhibitors may turn out to be therapeutically valuable and inhibitor combinations may provide synergistic effects. Although specificity is usually sought to minimize side-effects, as pointed out by Goldberg, sometimes adjusting the dose of a non-specific drug can provide the requisite specificity. For example, inhibiting proteasome function by 40% has a small effect on most proteins, but significantly affects a subset of proteins with a high turnover rate.

Goldberg also stressed the importance of investigating the role of proteasomes in huntingtin processing and the effects of long poly-glutamine stretches. As recently reported by Neil Bence and colleagues, aggregation of a mutated huntingtin fragment can directly impair the function of the ubiquitin-proteasome system. But proteasomes are also likely to play a key role in the breakdown of huntingtin and its fragments. For example, Trottier described that 24 hours after inducing NG108 cells to express a caspase-3 fragment of mutated huntingtin, he was able to detect the previously described 55kDa fragment only when he blocked proteasome activity. This result suggests the fragment is generated early on, but is quickly cleared by proteasomes in the early stages of disease (after 18 days of expression, the fragment is readily identified without inhibiting proteasomes).

On the other hand, proteasomes could also contribute to the generation of huntingtin fragments. Goldberg explained that sometimes the threading of a protein through the proteasome stops before the whole protein has gone through, resulting in the release of an undigested fragment. In the case of NFκB, for example, this process seems to generate an active fragment. Although the phenomenon has so far been described for only a handful of proteins, Goldberg suspects it may represent an important new form of protein processing.

Some participants suggested searching for altogether new proteases. Dröge, for example, described looking for proteases that cleave huntingtin upstream of its caspase sites. Reasoning that shorter fragments aggregate more readily, Dröge and her colleagues used a filter-based assay to screen for increased production of aggregates in a cDNA library of cells transfected with a 500-amino acid huntingtin construct. The researchers have so far identified several clones, but none of them has shown increased protease activity. Dröge suspects that the increased aggregation resulted from an elevation in huntingtin expression, or an alteration of chaperone function. She is now planning to set up a screen in yeast using cell growth as an indicator of proteolytic activity.

### ***Tracking protease expression and activity***

Several methods were discussed to monitor protease expression and activity. To compare the expression of proteases in HD and control samples, for example, Hook is using cDNA microarrays. She reported that between 400 and 500 protease genes have been identified using an array sporting 40,000 human cDNA sequences. Now Hook plans to focus on these genes using smaller arrays carrying exclusively protease genes.

Fluorescence-based assays surfaced as powerful tools to track protease activity. Stack and his colleagues at Aurora Biosciences, for example, are developing an assay to monitor the cleavage of huntingtin and its ability to get into the nucleus by using an exon 1 construct fused to a nuclear export signal on one side, and to a transcription factor that activates the expression of a beta-lactamase gene on the other. Only when exon 1 is cleaved, and thus separated from the export signal, can the transcription factor move into the nucleus and activate production of the enzyme whose activity can then be tracked by the generation of a fluorescent product. Searching for compounds that block this activation could yield new protease inhibitors, as well as inhibitors of nuclear transport.

Approaches using fluorophores directly attached to huntingtin were also discussed. As noted by Dale Bredesen, for example, positioning a fluorophore and a quencher at different sites along the length of huntingtin could help reveal when two regions of the protein become separated by cleavage. Reisine added that several pharmaceutical companies, such as Glaxo, are using fluorescent probes to track the clipping of proteins and the subcellular location of fragments within cells. One potential problem with labeling huntingtin directly, however, is that the fluorophores could affect the conformation, and consequently the proteolysis and function, of the protein. In addition, as noted by Li, some fluorophores, such as GFP, interfere with fragments' transport into the nucleus.

As previously mentioned, the activity of proteases can also be followed using the suicide substrates described by Bogyo. A particularly powerful approach may result from performing experiments in parallel to monitor huntingtin cleavage status and enzyme activity.

### **The context of huntingtin proteolysis**

There is more to understanding huntingtin proteolysis, however, than identifying huntingtin's proteolytic fragments and the proteases that generate them. Elucidating their physiological role will require pinpointing where and when they act.

### ***Cell specificity***

One of the fundamental questions in HD research is understanding its cellular specificity. Huntingtin protein is expressed broadly, as are the caspases that have been suggested to be key for its processing. Yet a small group of cells in the striatum, the medium spiny cells, seem to sustain by far the most damage. One possibility, pointed out by Thurmond, is that susceptibility may be determined by a cell's regulation of caspase activation. Alternatively, or additionally, as noted by Goldberg, variations in cells' activation of heat shock responses may determine vulnerability.

But the problem of specificity may be more complex. As noted by DiFiglia, a recent study using confocal microscopy to examine the distribution of huntingtin among striatal projection neurons, revealed that only 27.6% of enkephalin-containing neurons --those most damaged in HD-- harbor detectable amounts of huntingtin. Other studies, also described by DiFiglia, indicate that the large cholinergic neurons in the striatum are relatively unaffected in HD, yet express huntingtin protein and harbor nuclear inclusions.

These data suggest that the death of the medium spiny cells may be caused through the effects of huntingtin on other neurons --what has been referred to as murder, as opposed to suicide. Indeed, as noted by Johnson, a recent report using R6/2 chimeric mice supports this possibility. By counting the R6/2 cells in various brain areas of mice with random mixes of wildtype and R6/2 cells, the researchers found no correlation between morbidity and the abundance of R6/2 neurons in the striatum and in the basal ganglia, in general. Surprisingly, they observed an inverse, linear relationship between the survival of R6/2 chimeras and the percentage of R6/2 cells in some nuclei of the thalamus and amygdala.

Regardless of whether the toxic effects of mutated huntingtin are direct or indirect, it will be important to elucidate the molecular mechanisms that lead to cell death. As noted by Trottier, in HD brains, the evidence for apoptosis is controversial, and in HD mice --including R6/2 mice, knock-in mice and YAC transgenic mice-- there is no obvious apoptosis. Dark cells are found in R6/2 and YAC mice, but the link between these cells and pathogenesis is not clear.

Dale Bredesen noted that morphological descriptions of apoptosis and necrosis are not adequate to describe all cell deaths. He described a non-apoptotic form of cell death, paraptosis, often seen in development and neurodegeneration, which is mainly characterized by cytoplasmic manifestations, such as vacuolation. As a first step towards examining the potential role of paraptosis in HD, Bredesen suggested studying cleavage of huntingtin during paraptosis in transfected cells.

### ***Subcellular localization***

Although the subcellular localization of huntingtin has been investigated in numerous studies, its importance in the disease remains uncertain. Mutant N-terminal fragments have long been known to form nuclear inclusions, and several researchers have suggested they are toxic. However, as noted by McMurray, some studies have shown that treatment of cells with caspase inhibitors increases both the incidence of nuclear inclusions as well as cell survival. Regardless of whether nuclear inclusions are toxic or protective, they are clear markers of disease, and thus, participants agreed, warrant further study.

As previously mentioned, immunostaining experiments have revealed that nuclear inclusions are different from cytoplasmic aggregates. As noted by DiFiglia, one source of this difference may be traced to a recently identified nuclear export signal in the C-terminus of huntingtin. Trottier also found that in NG108 cells expressing N-terminal huntingtin, fragments

with normal repeats stayed in the cytoplasm, whereas those with expanded repeats accumulated in the nucleus. Li added that his group has obtained similar results with PC12 cells, and that treating the cells with leptomycin B, an inhibitor of nuclear export, resulted in the nuclear accumulation of both fragments. Although subject to the limitations of immunostaining, these data suggest that while both wildtype and mutant fragments enter the nucleus, only wildtype pieces get shuttled out.

An intermediate step in the journey of huntingtin fragments to the nucleus was described by Sawa. Working with human lymphocytes treated with staurosporine to induce stress and the production of caspases, Sawa found that 5 to 10 hours after staurosporine treatment, N-terminal huntingtin fragments accumulated in specific peri-nuclear regions. He hypothesized that this might be a site where further proteolytic processing occurs.

The localization of huntingtin fragments to cell membranes may also be an important part of the disease process. DiFiglia suggested that much of the proteolytic processing of huntingtin may occur at membranes. Based on her recent results, she proposed that caspase 3 cleavage may result in the association of N-terminal huntingtin fragments with membranes where they are further processed by calpains. Calpain-induced cleavage may then lead to the formation of mutant huntingtin fragments that can aggregate and form inclusions. In support of calpains' role in HD, Ellerby reported that calpain activity is increased in HD brains compared to controls.

### ***Timing***

Assuming that huntingtin processing is important for disease progression, elucidating the timing of each cleavage will be critical for defining a causal chain of events. Consider, for example, the importance of timing in understanding the role of calpains. One possibility is that calpain processing of mutant huntingtin is an early event, generating toxic fragments that mediate cell dysfunction. Another possibility is that calpains cleave huntingtin after the primary toxic insult has occurred –i.e., the enzymes could become active in response to the surge in calcium that accompanies cell death. Yet another possibility, pointed out by Goldberg, is that calpain cleavage occurs even later: after harvesting the tissue. Proteins derived from tissue samples are notoriously susceptible to calpain proteolysis because of the calcium leakage and the membrane disruption that often occur during manipulations such as freezing and thawing. Although this last scenario is unlikely to explain DiFiglia's results since her samples were immediately frozen after dissection, and remained so until they were exposed to protease inhibitors, it may apply to other studies.

Timing is also important because a cleavage made by one protease may depend on previous cleavages made by other proteases. Drawing on his experience studying the processing of pro-opiomelanocortin and proinsulin by proteases PC1 and PC2, Cawley stressed that it was important to determine the *in vivo* pathway of huntingtin cleavage. He cautioned that *in vitro* experiments could be misleading because, given the right conditions, many proteases can cleave target sites, obscuring their *in vivo* dependence on previous cleavages. Thus, he and other participants proposed performing pulse-chase experiments. Cawley added that conditions that trigger apoptosis, inhibit caspases, or inhibit proteasomes could be applied to assess their effects on the metabolic processing of normal huntingtin.

As noted by Trottier, the temporal relationships between the generation of fragments, aggregate formation, the alteration of proteasome function, and the disruption of neural function are also critical, yet remain unknown. One possibility, suggested by Goldberg, is that pathogenesis occurs as a two-step process in which cellular stress accelerates the expression of a

genetic susceptibility. Supporting this idea, Sawa described his observations with HD lymphocytes indicating that mutant cells' propensity to die was only revealed in the presence of a stress-inducing agent such as staurosporine. Ellerby added that the analysis of gene expression using microarrays has revealed that mouse models of HD show increased expression of several heat shock protein genes.

Working with HD knock-in mice generated by Peter Detloff and a set of well-characterized antibodies, Li said his group is planning to examine how nuclear aggregates change with time. Using antibody EM121, for example, he hopes to determine whether the intranuclear aggregates of younger mice are stained more strongly than those of older mice suggesting that more N-terminal huntingtin fragments accumulate in the nuclei of older neurons.

### ***Model systems***

An issue that surfaced repeatedly at the workshop was the selection of model systems. As previously described, *in vitro* systems, such as the use of brain extracts to study proteolysis, were favored by some participants because of their accessibility to biochemical manipulations. However, the inability of these systems to accurately mirror *in vivo* conditions was noted as an important limitation. Participants also discussed the use of subcellular fractionation to study isolated inclusions. For example, Tobin proposed analyzing the composition of inclusions using mass spectrometry. Goldberg noted, however, that it will be important to establish isolation guidelines to draw reproducible distinctions between inclusion components and their associated proteins.

Many stressed the value of cell-based assays for testing basic hypotheses, as well as for screening candidate drugs. Terry Reisine, for example, noted the usefulness of these assays in studies of Alzheimer's disease. He noted that several methods for high throughput screening of cells have been recently developed using multi-well plates. DiFiglia mentioned that her group is setting up a cell-based assay to screen for compounds that block caspase and calpain cleavages.

Selecting a worthy cell model, however, is not easy. As noted by DiFiglia, overexpression of huntingtin can lead to an over-activation of caspases, and may also affect normal nuclear transport, as suggested by Sawa. In addition, overexpression may cause abnormal cellular behaviors such as the secretion of huntingtin into the media, as described by DiFiglia. On the other hand, too low of an expression can make the protein hard to detect. Wexler suggested that inducible models, such as the mouse model generated by Ai Yamamoto, may help circumvent at least some of these problems.

Another consideration is the selection of cell type. Sawa favored using lymphocytes from patients because they are human, can be grown in culture, and express huntingtin at physiological levels. Bredesen added that non-neuronal cells may be particularly good models because of their hardiness. Several studies have shown that non-neuronal cells in a variety of neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, and several poly-glutamine disorders, behave abnormally when stressed. It is thus possible that these cells are subject to the same disease processes that afflict neurons, but experience them in a milder form that is more easily studied in culture.

Nevertheless, most participants favored using neuronal cells to match the target of the disease. McMurray and Hook stressed that cells differ in the proteases they express and activate, so it is important to work with neuronal cells to focus on the cleavages that are more likely to be relevant to the disease. Goldberg added that ubiquitination and permeability to small molecules can also differ greatly between cell types. In addition, McMurray noted that the permeability of

the nuclear membrane varies –some cells allow larger molecules through their nuclear pores than others.

Unfortunately, noted Reisine, primary neurons are difficult to maintain and transfect. So an alternative supported by many participants was the use of neuronal-like cell lines --in particular, NG108 cells, derived from the fusion of a neuroblastoma and a glioma cell line. As described by Trottier, NG108 cells can be readily grown in culture and then differentiated into neuronal-like, non-dividing cells that can be maintained for at least 18 days. According to his studies, the huntingtin fragments generated by these cells correspond well to those found in HD brains. He cautioned, however, that although these cells were good models for studying proteolysis, they may not be ideally suited for studying toxicity because their differentiation involves cAMP stimulation.

Participants also discussed the selection of animal models for *in vivo* studies. As explained by DiFiglia, animal models carrying the full-length huntingtin gene, such as knock-in mice, mirror HD more faithfully than models overexpressing a short fragment of huntingtin, such as R6/2 mice, because the damage is more limited to specific cell types, and the disease develops more slowly. McMurray added that knock-in mice show less cleavage of huntingtin, and less inclusions, characteristics which better match adult-onset HD.

However, several participants defended the use of models bearing short fragments. As noted by DiFiglia, these models are more clear-cut and less variable in their expression of pathology and symptoms facilitating experimental analysis. In addition, said Goldberg, the short-fragment models progress through the disease more rapidly, enabling the performance of more experiments per unit time. It has yet to be determined, however, whether the short-fragment models are indeed expressing a sped-up form of HD, or alternatively, suffering from a disorder with an altogether different mechanistic basis. As proposed by Minka vanBeuzekom, an alternative to creating accelerated models of HD might be to cross knock-in mice with mice used as models for premature aging disorders, such as Werner's syndrome.

Given the lack of detailed comparisons of pathological progression in different animal models of HD, especially in the early stages of the disease, Li proposed organizing a workshop focusing on the formation of inclusions and cellular pathological alterations, including cell body degeneration, increased expression of glial fibrillary acidic protein (GFAP), and neuritic atrophy. Li considered these changes to be valuable indicators of disease because they are more easily quantified and less affected by genetic backgrounds than behavioral alterations.

## **Therapeutic alternatives**

The number of uncertainties in the study of huntingtin processing is so large that, in many ways, it seems premature to begin considering therapeutic options based on the inhibition of proteolysis. As Hersh pointed out, how does one design a drug without a target? It is still unclear, as noted by Cawley, whether in the absence of proteolytic processing, mutant huntingtin is more or less toxic, or the onset of disease is slower or faster.

Nevertheless, participants agreed that at least some approaches are worth beginning to explore. As noted by Goldberg, even if huntingtin proteolysis proves to be of only minor importance in mediating HD pathology, interventions that target proteases, in particular caspases, may be of therapeutic value because of their potential to curb apoptosis. Goldberg also considered it worth examining these options without being overly concerned about the inhibitors' potential side-effects. He noted that a drug's toxicity is often hard to predict. Referring to his

experience with proteasome inhibitors to treat cancer, now in phase II clinical trials, Goldberg noted that sometimes drugs do not live up to their expected toxicity. In addition, even if protease inhibitors have some side-effects, they may be acceptable given HD's lethality.

Participants also discussed therapeutic alternatives to protease inhibitors. Hersh, for example, suggested searching for an enzyme in microbes that cleaves within large poly-glutamine stretches. Although abnormal huntingtin is not the only protein containing poly-glutamine stretches, noted Hersh, it may be possible to derive an enzyme specific for the huntingtin stretch.

Participants also suggested examining treatments that upregulate proteasomes and the heat shock response. McMurray, for example, is testing the effects of a derivative of geldanamycin --a compound that binds to the heat shock protein Hsp90 and activates a heat shock response in mammalian cells-- on R6/2 cells. A recently published study by Erich Wanker and colleagues showed that treatment of cells with geldanamycin induces the expression of Hsp40, Hsp70 and Hsp90 and inhibits huntingtin exon 1 aggregation in a dose-dependent manner. Goldberg added that exposing cells to brief pulses of proteasome inhibitors can also be used to induce a heat shock response and bolster a cell's abilities to cope with stress.

### Summary

The workshop provided a fruitful forum for the discussion of the relevance of proteolysis to HD pathogenesis. Perhaps more importantly, it generated a list of ideas for future research. Participants agreed that the identification and characterization of huntingtin fragments was a priority, as well as the identification of associated proteases. They also underscored the importance of tracking huntingtin processing *in vivo*, examining the order of cleavage, and assessing the importance of fragment subcellular localization. Although much remains to be done before huntingtin processing can provide a strong basis for rational therapy design, a path towards this worthy goal is coming into focus.

### List of Action Items:

1. Identify proteolytic fragments: Antibody-based strategies
  - a. Standardize gel electrophoresis and Western conditions (Foundation to set up guidelines?)
  - b. Conduct epitope mapping for key antibodies (Li, Trottier)
  - c. Generate additional antibodies for further fragment characterization (Ellerby, Sawa, Li)
  - d. Compare wildtype and mutant cleavages under various conditions *in vitro* (Cawley, Li)
2. Identify proteolytic fragments: Sequence, secondary modifications, and structure
  - a. Use mass spectrometry to identify fragment sequences and secondary modifications (Gibson, Sawa, Hook, Trottier)
  - b. Apply limited proteolysis and cross-linking to examine fragment structure (Gibson)
  - c. Perform X-ray crystallography to determine fragment structure (McMurray)
3. Identify relevant proteases: genetic-based assays
  - a. Generate animals bearing point mutations in huntingtin cleavage sites (Wellington)
  - b. Generate dominant-negative mutants for specific proteases (Ellerby)

4. Identify relevant proteases: protease inhibitors
  - a. Obtain and test new protease inhibitors developed by companies (DiFiglia, Goldberg, Foundation)
  - b. Develop suicide substrates for caspases and calpains (Bogyo)
  - c. Test general protease inhibitors (various)
  
5. Search for new proteases
  - a. Use yeast to screen for new proteases (Dröge)
  - b. Assess proteasome's capability to produce huntingtin fragments (Goldberg)
  - c. Perform biochemical purifications and enrichments using high-performance liquid chromatography (Li)
  
6. Track protease expression and activity
  - a. Use microarrays to assess protease expression (Hook)
  - b. Track huntingtin cleavage using fluorescent emitter and quencher partners (Bredesen)
  - c. Use suicide substrates to create roadmap of protease activation (Bogyo)
  
7. Assess the context of huntingtin processing
  - a. Analyze cleavage during paraptosis (Bredesen)
  - b. Examine fragment transport into the nucleus (Sawa)
  - c. Assess role of cell membranes in huntingtin processing (DiFiglia)
  - d. Perform pulse-chase experiments to elucidate sequence of cleavages (various)
  - e. Track changes in fragment composition of inclusions with age (Li)
  
8. Improve model system selection
  - a. Consider adopting neuronal-like cell lines (e.g., NG108) for cell-based assays of huntingtin processing (various)
  - b. Cross HD mice with mice that age prematurely (vanBeuzekom)
  - c. Organize workshop on mouse models (Li, Foundation)
  
9. Begin explorations of potential therapeutic candidates
  - a. Use cell-based assay to screen for caspase and calpain inhibitors (DiFiglia)
  - b. Use cell-based assay to screen for compounds that inhibit proteolytic cleavage and/or nuclear transport (Stack/Aurora Biosciences)
  - c. Search for enzyme in microorganisms that cleaves within poly-glutamine stretches (Hersh)
  - d. Test effects of upregulating proteasomes and heat shock response (McMurray, Goldberg, Foundation)

## References

Bence, NF; Sampat, RM; Kopito, RR. Impairment of the ubiquitin-proteasome system by protein aggregation. [Comment In: Science. 2001 May 25;292(5521):1467-8 UI: 21269743] Science 2001 May 25, 292(5521):1552-5.

Cooper, J. K., Schilling, G., Peters, M. F., Herring, W. J., Sharp, A. H., Kaminsky, Z., Masone, J., Khan, F. A., Delanoy, M., Borchelt, D. R., Dawson, V. L., Dawson, T. M. and Ross, C. A. (1998). Truncated N-terminal fragments of huntingtin with expanded glutamine repeats form nuclear and cytoplasmic aggregates in cell culture. *Hum Mol Genet* 7, 783-90.

Deng, Y.P, Xie, J.P., Del Mar, N., Meade, C.A., Goldowitz, D., Reiner, A. The neural basis of morbidity in the R6/2 chimeric mouse. Soc. Neurosci. Abstr., Vol. 27, Program No. 863.8, 2001

DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P., Aronin, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* 277, 1990-1993.

Dyer, RB; McMurray, CT. Mutant protein in Huntington disease is resistant to proteolysis in affected brain. *Nature Genetics*, Nov, 2001, V29(N3):270-278.

Fusco, F.R., Martorana, A., Longone, P., Guatteo, E., Viscomi, M.T., Sancesario, G, Molinari, M., Bernardi, G. Huntingtin distribution among striatal projection neurons: a confocal microscopy study. Soc. Neurosci. Abstr., Vol. 27, Program No. 69.15, 2001

Goldberg, YP; Nicholson, DW; Rasper, DM; Kalchman, MA; Koide, HB; Graham, RK; Bromm, M; Kazemi-Esfarjani, P; Thornberry, NA; Vaillancourt, JP; Hayden, MR. Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract [see comments] *Nature Genetics* 1996 Aug, 13(4):442-9

Hackam, A. S., Singaraja, R., Zhang, T., Gan, L. and Hayden, M. R. (1999). *In vitro* evidence for both the nucleus and cytoplasmic subcellular sites of pathogenesis in Huntington's disease. *Hum Mol Genet* 8, 25-33.

Kim, YJ; Yi, Y; Sapp, E; Wang, YM; Cuiffo, B; Kegel, KB; Qin, ZH; Aronin, N; DiFiglia, M. Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis.

Li, H., Li, S-H., Johnston, H., Shelbourne, P., Li, X-J. Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. *Nature Genetics*, 25, 385-389, 2000

Lunkes, A. and Mandel, J. L. (1998). A cellular model that recapitulates major pathogenic steps of Huntington's disease. *Hum Mol Genet* 7(9), 1355-61.

Mangiarini L, Sathasivam K, Seller M, Cozens B, Harper A, Hetherington C, Lawton M, Trotter Y, Leach H, Davies SW, Bates GP. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell*. 1996 Nov 1;87(3):493-506.

Martindale, D., Hackam, A., Wiczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S. U., Bredesen, D. E., Tufaro, F. and Hayden, M. R. (1998). Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. *Nat Genet* 18(2), 150-4.

Mende-Mueller, LM; Toneff, T; Hwang, SR; Chesselet, MF; Hook, VY. Tissue-specific proteolysis of Huntingtin (htt) in human brain: evidence of enhanced levels of N- and C-terminal htt fragments in Huntington's disease striatum. *Journal of Neuroscience* 2001 Mar 15, 21(6):1830-7.

Schilling G, Wood JD, Duan K, Slunt HH, Gonzales V, Yamada M, Cooper JK, Margolis RL, Jenkins NA, Copeland NG, Takahashi H, Tsuji S, Price DL, Borchelt DR, Ross CA. Nuclear accumulation of truncated atrophin-1 fragments in a transgenic mouse model of DRPLA. *Neuron* 1999 Sep;24(1):275-86

Sittler, A., Lurz, R., Lueder, G., Priller, J. Lehrach, H., Hayer-Hartl, M.K., Hartl, F.U., Wanker, E.E. Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease. *Hum. Mol. Genet.* 2001 Jun 1,10, 1307-1315.

Waelter, S., Boeddrich, A., Lurz, R., Scherzinger, E., Lueder, G., Lehrach, H., Wanker, E.E. (2001) Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol. Biol. Cell* 12, 1393-1407.

Wellington, CL; Singaraja, R; Ellerby, L; Savill, J; Roy, S; Leavitt, B; Cattaneo, E; Hackam, A; Sharp, A; Thornberry, N; Nicholson, DW; Bredesen, DE; Hayden, MR. Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. *Journal of Biological Chemistry* 2000 Jun 30, 275(26):19831-8.

Wellington, CL; Ellerby, LM; Hackam, AS; Margolis, RL; Trifiro, MA; Singaraja, R; McCutcheon, K; Salvesen, GS; Propp, SS; Bromm, M; Rowland, KJ; Zhang, T; Rasper, D; Roy, S; Thornberry, N; Pinsky, L; Kakizuka, A; Ross, CA; Nicholson, DW; Bredesen, DE; Hayden, MR. Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *Journal of Biological Chemistry* 1998 Apr 10, 273(15):9158-67

Wheeler VC., White JK. Gutekunst CA., Vrbanac V. , Weaver M. Li XJ. Li, SH. Yi, H., Vonsattel JP., Gusella JF., Hersch S., Auerbach W. , Joyner AL, MacDonald ME. Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. *Human Molecular Genetics.* 9(4):503-13, 2000

## **Participants**

Mathew Bogyo  
University of California, San Francisco

Dale Bredesen  
UCSF Buck Institute for Age Research

Niamh Cawley  
NIH-NICHD

Marina Chicurel  
Science Writer

Marian DiFiglia  
Massachusetts General Hospital

Anja Droege  
MPI for Molekulare Genetics

Lisa Melinda Ellerby  
Buck Institute for Age Research

Bradford Gibson  
Buck Institute for Age Research

Alfred Goldberg  
Harvard Medical School

Louis Hersh  
University of Kentucky

Vivian Hook  
Buck Institute for Age Research and  
University of California, San Diego

Carl Johnson  
Executive Director, Cure HD Initiative

Blair Leavitt  
University of British Columbia

Xiao-Jiang Li  
Emory University

Cynthia McMurray  
Mayo Clinic and Foundation

Terry Reisine  
ActiveSite Biotech

Christopher Ross  
Johns Hopkins University School of Medicine

Akira Sawa  
Johns Hopkins University School of Medicine

Jeffrey Stack  
Aurora Biosciences Corporation

Robin Thurmond  
R.W. Johnson Pharmaceutical Research Institute

Allan Tobin  
UCLA Brain Research Institute and  
Hereditary Disease Foundation

Yvon Trottier  
IGMC CNRS Université Louis Pasteur

Minka vanBeuzekom  
Cure HD Initiative

Cheryl Wellington  
University of British Columbia

Nancy Wexler  
Columbia University and Hereditary Disease Foundation