

Hereditary Disease Foundation  
**“Cure Hunters Reunion Workshop”**

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Hereditary Disease Foundation  
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The “Cure Hunters Reunion Workshop” was a special meeting. It provided a forum for the insightful discussion of new ideas, as well as current challenges and successes in the field of Huntington’s disease (HD) research. It also provided a rare and unique view of the scientific and human efforts that led to the birth of modern-day HD research.

Following a fascinating conversation about the long road HD researchers have traveled over the past four decades with support from the Hereditary Disease Foundation, participants discussed how to keep moving the field forward. They brainstormed new ways to use the vast HD tissue collections and datasets that have been painstakingly built over the years. The use of sophisticated bioinformatics tools was suggested, for example, to potentially uncover distinct forms of HD with distinct patterns of disease progression. Participants also discussed the current status and future of approaches to search for genetic modifiers of HD. They analyzed the limitations of past searches, potential reasons for the current lack of validated candidates, and new possibilities for performing more effective searches, including looking beyond the genome to the epigenome.

Participants also discussed several promising new therapeutic targets. In particular, various post-translational modifications emerged as encouraging possibilities. For example, participants described the recent identification of the negative regulator of ubiquitin-like protein 1 (NUB1) as a protein involved in enhancing mutant huntingtin degradation by proteasomes. Also, PIAS-1, a protein involved in the SUMOylation of huntingtin and its degradation, was discussed as a promising therapeutic target. Moreover, participants were encouraged by a new opportunity for targeting neuroinflammation using MIND4, a novel lead compound which appears to be neuroprotective in various models of HD.

Possibilities to improve one of the most advanced candidate therapies in the HD pipeline, gene silencing, were also discussed. For example, the use of manganese nanoparticles as carriers to deliver silencing agents throughout the brain emerged as an intriguing new option to increase silencer distribution and reduce invasiveness. In addition, participants discussed ways to enhance silencing approaches based on new findings indicating the generation of an aberrantly spliced huntingtin fragment in HD.

### **Looking back and looking forward**

Bringing together many of the scientists involved in the search, discovery, and early characterization of the huntingtin gene, the meeting began with researchers reminiscing about the long road they have traveled with support from the Hereditary Disease Foundation. The story that emerged was an inspiring collection of individual accounts from researchers who, after years of hard work, are still committed to helping find a cure for HD.

There were several recurring themes in the stories that were shared: the dogged persistence of scientists in the face of huge challenges, humorous anecdotes exposing the human side of arduous years of research, and perhaps most strikingly, the inspiration and

support that the Hereditary Disease Foundation, and in particular its president Nancy Wexler, have provided HD researchers throughout the years.

One of the early challenges, noted Thomas Chase, was the scarcity of high quality researchers in the field. In 1972, working with N. Wexler and others, he helped organize a large international symposium to which leaders in research disciplines that could be important for HD were invited. Most of the participants had never actually worked on HD, said Chase, but, as hoped, many were tempted to apply their technologies to have some results for presentation at the meeting. Chase considers that this meeting, and others to follow, served as a stimulus for HD research.

As the field moved forward, the importance of identifying the HD gene and its mutation to develop treatments and cures became increasingly apparent. So, as described by N. Wexler, in 1979, the Hereditary Disease Foundation organized a workshop to explore the idea of mapping the gene using restriction fragment polymorphisms (RFLPs). RFLPs had just been recently identified as potentially useful DNA markers, however, and only a handful of them had been mapped. Thus, the consensus at the workshop was that it would take decades to find sufficient RFLPs and position them on chromosomes to fully map the human genome. Only after accomplishing this would it then be possible to start searching for the HD gene. In addition, the researchers pointed out that it would be nearly impossible to find a large enough family to conduct a fruitful search.

Despite all this, a young molecular biologist, David Housman, boldly suggested starting the search for the HD gene right away. He acknowledged that it might take several decades to find a marker that co-segregated with the gene, but he also reasoned that if one of the early-found markers was linked to the gene, they might get lucky and map the gene sooner than expected. Housman also suggested that as new DNA markers emerged, they could be tested for co-segregation. Most participants at the workshop considered Housman's idea premature. They worried that recruiting families for the study would get their hopes up for a project that could take decades, possibly even 100 years.

Nevertheless, Housman and colleagues pursued the idea and wrote a grant to the NIH, as well as a proposal asking for seed money from the Hereditary Disease Foundation. It was a hectic time for Housman. Not only was he pushing hard for a controversial idea, but his wife was about to deliver a baby. And to top it all off, the day before he was going to present his idea, he got sick. Somehow he got himself to the meeting the next day and croaked out his proposal.

With unique vision, the Hereditary Disease Foundation funded Housman's grant. But still many challenges remained. Where could they find a family large enough to conduct the mapping study? Work on another genetic disease, familial hypercholesterolemia, had shown that studying homozygotes was extremely valuable for understanding genetic disorders. So in search of HD homozygotes, N. Wexler had travelled, just months before the 1979 workshop, to the Lake Maracaibo region in Venezuela where Venezuelan scientists had identified a community with a very high prevalence of HD. After the

workshop, Wexler and her colleagues realized that the Lake Maracaibo families would not only be helpful in the search for homozygotes, but also in the search for the HD gene.

The identification of these unique families was a very important milestone. But the team needed money to do the required work. N. Wexler could not let this bump in the road slow them down. So, in her characteristic fashion, she took it upon herself to solve the problem. As Housman said, “Nancy somehow got the cash to do it!” The Hereditary Disease Foundation received a grant from the National Institute of Neurological Disorders and Stroke (NINDS), NIH. This grant paid for research in Venezuela, including travel, tissue and pedigree collection and examining families in this very large kindred. A separate NIH grant called “Center Without Walls” got funded for Massachusetts General Hospital to do linkage analysis.

To everyone’s surprise, in just three years, the team discovered a marker that co-segregated with the HD gene. The mapping project that some believed could take 100 years had yielded results in a mere 36 months. Gusella, N. Wexler, and the team were studying a large American family with HD and the twelfth RFLP they tested yielded a positive result—a logarithm of the odds ratio (LOD) score of 1.81. A LOD score is a statistical measure of the likelihood of two genetic loci residing near each other on a chromosome so that they are inherited as a package, or linked. Furthermore, analysis of the Venezuelan families yielded a LOD score of 6.72. Adding the two scores together, the researchers obtained a composite LOD score of 8.53—proof of nearly a billion to one odds that the HD gene is at the top of chromosome 4. (By convention, a LOD score greater than 3.0, indicating that the odds are a thousand to one, is considered evidence for linkage). Not only had the team succeeded in mapping the HD gene, but they had proved that the gene mapping strategy worked—an accomplishment that would pave the way for the Human Genome Project and the mapping of many other hereditary diseases and genetic traits.

Pinpointing the HD gene proved more difficult, however, but was finally accomplished ten years later, in 1993. After that, came the search for the molecular and cellular mechanisms underlying the disease, a surprisingly challenging problem that is still underway. Key to all these efforts, including the initial mapping of the HD gene, were annual trips to Venezuela by a group of interdisciplinary scientists led by the Hereditary Disease Foundation from 1979 to 2002. Many of these scientists were at the Cure Hunters Reunion Workshop and, as they recounted their experiences, a picture of persistence, camaraderie, and inspiration emerged.

As described by several participants, the trips to Venezuela involved traveling to several extremely poor fishing villages along the shores of Lake Maracaibo, gathering oral pedigree information, performing neuropsychiatric examinations, and collecting blood, sperm and skin samples. Transformed lymphocyte lines and skin fibroblast lines were then created back in the U.S. As noted by Housman, at first, the throughput was very low and the researchers had to invest a lot of effort in each collected sample. McDonald added that freezing and keeping the cells alive was challenging too. One time, noted Anne Young, the sample boxes arrived in the U.S. full of red ants.

Several participants remembered the small jobs they helped out with and joked about their contributions to the research. For example, Jang-Ho Cha said he did all the heavy lifting and was the driver who brought food to the other scientists, while Ira Shoulson said he was in charge of sample labeling. Gustavo Rey said his role was to make sure the other researchers didn't make blunders when attempting to speak Spanish. Indeed, William Yang recalled the need for Rey's linguistic help when he mistakenly told a little girl "no te cagas" (don't defecate), instead of "no te caigas" (don't fall).

Other mishaps included Young's conflict with the photographer commissioned to record the team's efforts. The photographer, a close friend of N. Wexler, complained that Young was not being helpful when he tried photographing her performing neurological exams. The conflict escalated and N. Wexler had to intervene to help the two get along.

Juan Sanchez-Ramos, another member of the Venezuela HD Project, had many colorful anecdotes to share. Sanchez-Ramos went to the Lake Maracaibo villages as an expert neurologist from the U.S. But unlike other scientists on the team, the area held special significance to him: he was born en route to Caracas, just outside Maracaibo. Little did he know that his research interests would take him back to his place of birth.

One of the experiences that Sanchez-Ramos shared was how several project members, including himself, N. Wexler, Young, and physician Margot de Young, went to a fortune teller called "La Bruja." Sanchez-Ramos asked whether he would find the cure for HD and La Bruja said he would. When he asked how or where, La Bruja responded with great confidence, "En una planta" (in a plant). After that, Sanchez-Ramos actually spent several years investigating plant-derived products, such as sedatives and other pharmacological agents. As described later in this report, he is now developing a potential delivery system for gene silencing agents, molecules which were designed based on the discovery of the siRNA system in plants.

Despite the diversity of anecdotes and perspectives presented at the meeting, a common theme kept resurfacing, tying these stories together: the inspiration and support provided by the Hereditary Disease Foundation, and N. Wexler, in particular. Some researchers described the unique opportunity of meeting and interacting with top-notch scientists. Rey, for example, noted his excitement to be working with "the rockstars of neurology" and Gillian Bates marveled at her chance to meet "the most amazing scientists." Ai Yamamoto emphasized how much she had learned from everyone in the room and how exciting it has been to be part of the HD research community. She said she had originally trained as a materials science engineer, but once she connected with the Hereditary Disease Foundation she wanted to stay in biological research.

Several others also talked about how they became part of the HD research community. For example, Chase said that within a year of meeting N. Wexler, he was working on HD, and Yang said that after meeting N. Wexler and going to Venezuela he was "hooked." Thompson described the HD research community assembled by the Hereditary Disease Foundation as a family to her. And administrator and Venezuela Project

Coordinator Julie Porter said she had been “sucked in” by the Hereditary Disease Foundation seventeen years ago and is still part of the HDF family.

Participants also commented on the inspiration they derived from the Hereditary Disease Foundation workshops. Bob Hughes, for example, said the first time he went to a workshop and saw scientists brainstorming together, he was “utterly captivated and amazed” by the process. He was also deeply moved by the participation of a woman in her 20s suffering from HD. When he returned to his lab, he immediately told the principal investigator, Stan Field, that he wanted to work on HD. Field wasn’t surprised and even said he had predicted this would happen. Thompson and Yamamoto were also very inspired by the workshops. “It changed the way I thought about the world,” Thompson said.

Alice Wexler’s book “Mapping Fate” was also inspirational to several of the attendees. The book tells the story of the Wexler family, their trials living with the risk of HD, and their success in spearheading the effort to map the HD gene. Joan Steffan said that “Mapping Fate” had a “profound influence” on her and was key to triggering her research interest in HD. Moreover, Thompson noted that she was also inspired by the book and went to A. Wexler’s signing of the book when she was a graduate student working on yeast genetics. There she met N. Wexler who invited her to Venezuela, an experience that “opened her eyes” and shaped her future career path.

After taking stock of how much they have been through and accomplished, participants turned their attention towards discussing the current understanding of HD’s mechanisms of pathology, the therapeutic candidates in the pipeline, and the strategies and approaches that are most needed to keep the search for a cure on target.

### **Aberrant splicing of mutant huntingtin mRNA**

As discussed at the January workshop, a fundamental new insight into the pathology of HD was recently revealed by Gill Bates and David Housman. They showed that mutant huntingtin mRNA is spliced in mice and humans, resulting in the production of an exon 1 fragment. The fragment’s production is dependent on CAG length and could play an important role in HD pathology, considering its well-known toxicity. The finding is important for several reasons. It provides a potentially new mechanism to explain the molecular underpinnings of HD, it helps clarify several puzzling observations from previous studies, and it may prove important for shaping current and future therapeutic strategies.

As summarized by Bates, she became interested in huntingtin fragments about a decade ago when she noticed that a knock-in mouse model of HD (Detloff, Q150) had a remarkably similar phenotype to her R6/2 mouse model, a transgenic carrying a genomic fragment spanning the 5’ end of the huntingtin gene, exon 1 with approximately 150 CAG repeats, and a portion of intron 1. Bates observed that the knock-in mice developed the HD phenotype more slowly than the R6/2 mice, but the changes in heat shock

response pathways, as well as several other pathologies, were nearly identical in the two models.

Consistent with these observations, an unbiased screen for mutant huntingtin fragments that drive HD pathology resulted in the identification of CAG-expanded exon 1 (as revealed by the neo-antibody MW8). When Bates examined the R6/2 construct carefully, she realized it was possible that an exon 1 fragment could be generated by aberrant splicing of the huntingtin message. To test this hypothesis, Bates and Housman set out to search for the putative spliced fragment. But as explained by Housman, the search proved very challenging. The start of intron 1 is extremely rich in GC content and, combined with the secondary structure adopted by the CAG repeat, it makes the boundary region between exon 1 and intron 1 very difficult to sequence. Indeed, this region of mouse huntingtin results in a “dead zone” in most RNA-Seq data.

One problem with high GC content is that sequence analysis software has trouble aligning these regions and discards them into the computational trash bin, noted Marcy McDonald. However, as explained by Housman, in this case, the problem was that the secondary structure was interfering with the PCR amplification step. Performing a large set of systematic tests, the team ultimately identified a couple of modifications to the PCR protocol that allowed them to consistently read through the region.

#### *Human data*

Analyzing HD fibroblast lines and postmortem brains, the team has found the mis-spliced fragment in humans as well. Initially, the researchers examined HD fibroblast lines derived from two individuals with juvenile HD and observed that the levels of huntingtin transcripts containing exon 1, the exon 1-exon 2 junction, and exon 2 were comparable to those found in control cells. In contrast, transcripts containing early intron 1 sequences were elevated in the HD cells compared to controls.

Subsequently, the researchers searched for the mis-spliced transcript in postmortem brain tissue. Although it was a very challenging task because of the high GC content and secondary structure described above coupled to the extreme loss of 5' end coverage characteristic of postmortem samples, the researchers succeeded in identifying the mis-spliced transcript. Using 3' RACE, the team analyzed RNA samples derived from the brains of three individuals with HD and found a huntingtin RNA fragment corresponding to the mis-spliced species of approximately 7,300 base pairs, as predicted from analysis of the human sequence, and seen in the BACHD and YAC128 mouse models that express the human gene.

It is important to note that the postmortem brain samples were derived from individuals with CAG repeat lengths between 40 and 75. One individual had 42 CAG repeats and experienced HD onset in his 40s. The other two suffered from early onset HD, but each had a relatively low number of CAG repeats (72) compared to the typical range of 80-100 repeats associated with juvenile HD. Thus, the findings cannot be attributed to a pathological process specifically associated with unusually long CAG repeats.

How much does the aberrantly spliced mRNA contribute to HD pathology?

As noted by Bates, determining the extent to which the newly identified transcript contributes to HD pathology is a top priority. Housman estimates that approximately 20% of mutant huntingtin transcripts in the Q150 knock-in mouse are aberrantly spliced. And because the fragment is highly toxic, this fraction could play a significant role in HD pathology.

To begin to examine this question, Bates is planning to generate mice that make full-length mutant huntingtin, but are not exposed to the mis-spliced exon 1 fragments. The idea is to create a huntingtin construct that lacks all the cryptic poly-A sites found in intron 1 so that any mis-spliced mRNAs made would transcribe through exon 2 and be degraded by nonsense-mediated. Bates cautioned, however, that little is known about the regulation and processing of huntingtin mRNA, and it is still uncertain if this strategy will work.

Unexpectedly, a transgenic mouse recently created by Scott Zeitlin offered what might be a preview of the results from this experiment. Zeitlin has been creating transgenic mice that express mutant huntingtin under the regulation of the Lac repressor system to explore how downregulating huntingtin production at different times during the course of HD affects disease pathology (see Gene Silencing: *Timing and magnitude*). However, by chance, one of the mouse lines incorporated two lac operators near the exon 1 5' splice site. The researchers observed that even in the absence of IPTG to downregulate huntingtin expression, these animals produced about 50% less huntingtin than controls. They also noticed that, surprisingly, at 15 months of age, these mice appeared to have no inclusions. Considering Bates's and Housman's recent findings, Zeitlin checked for the presence of the aberrantly spliced fragment reasoning that the lac operators might be interfering with splicing. Indeed, using RT-PCR and Western blots, Zeitlin found no sign of the mis-spliced exon 1 mRNA or protein in these mice.

The results are exciting because they may lend support to the importance of the aberrant fragment in HD pathology. However, Zeitlin strongly cautioned that they are preliminary and he has yet to examine other aspects of the HD phenotype, including behavior. Furthermore, because the experiment wasn't designed to test the toxicity of the fragment, there are a couple of caveats that complicate the interpretation of the results. The reduced levels of huntingtin expression make it impossible to cleanly compare the pathology and symptoms of the fragment-free mice to standard HD mice. It is also difficult to compare the fragment-free mice to mice with similar amounts of mutant huntingtin expression regulated by the lac operon system, noted McDonald, since the fragment-free mice will have experienced low levels of huntingtin, not only during postnatal life, but throughout development.

As another possibility to gain insight into the contribution of the mis-spliced fragment to HD pathology, Zeitlin suggested crossing mice expressing a cDNA construct of mutant huntingtin (which cannot be spliced) with mice expressing an exon 1 fragment. McDonald noted that her team did this experiment already and found that the effects on inclusion formation appeared to be additive, not synergistic. However, McDonald also



emphasized that it is possible they missed a synergistic effect, particularly because they did not monitor other parameters beyond inclusions.

McDonald wondered whether Shortstop mice, which carry a truncated human huntingtin construct terminating at intron 2, could provide additional insights. In the original report describing these mice, it was noted that Shortstop mice develop robust inclusion pathology without developing phenotypic abnormalities seen in other HD models. Could these mice be lacking the exon 1 mis-spliced fragment? It is possible that the Shortstop construct does not generate a mis-spliced fragment, although it would be expected to, given that it includes both exon 1 and intron 1. In addition, searching for the mis-spliced fragment in these mice may not be very informative because, as pointed out by Bates, their phenotype is likely due to anomalous levels and/or patterns of the construct's expression. Follow up studies by David Borchelt and others to the initial Shortstop publication demonstrated that transgenic expression of an equivalent fragment of huntingtin does, in fact, produce neurologic abnormalities similar to previously described HD mouse models.

Participants also briefly discussed ways by which the mis-spliced fragment could cause toxicity. As previously noted, it is well known that the exon 1 protein fragment is very toxic, but is the new finding of the mechanism by which it is generated relevant to its toxicity? Ai Yamamoto, for example, wondered if differences in splicing between cell types could help explain the differential vulnerability of various cells. However, Bates said that, so far, there doesn't seem to be any significant difference between tissues.

Another possibility suggested by McDonald is that the mutant huntingtin transcript could behave like a toxic mRNA. Housman pointed out in the January workshop, however, that the splicing alteration in HD must be fundamentally different from what occurs in other toxic mRNA diseases such as myotonic dystrophy because the huntingtin mutation appears to act only in cis, not trans.

The subcellular localization of huntingtin mRNA and how it might play a role in its toxicity was also discussed. As noted by McDonald, huntingtin mRNA has been detected at synapses. And it is possible that regulation and/or processing of the message, including splicing, as noted by Marina Chicurel, occurs locally at synapses. To explore this possibility, McDonald suggested examining mutant huntingtin mRNA's association with splicing factors by RNA-Binding Protein Immunoprecipitation (RIP) in synaptosomes. Hughes added that RNA Seq analyses at the synapse could also be performed. Moreover, Marian DiFiglia noted that other aspects of synaptic mRNA regulation, including activity-dependent transport and translation, could be examined by monitoring huntingtin mRNA's association with mRNA binding proteins such as FMRP.

At the level of the whole organism, Marie-Francoise Chesselet pointed out that she is interested in determining whether the exon 1 fragment is contributing to the dramatic differences in disease onset observed in various animal models of HD and how different huntingtin constructs affect fragment generation and stability. Moreover, Zeitlin noted

that he plans to investigate whether the fragment plays a role in normal cell function. Although fragment production is very low in wildtype cells, a small amount is detectable.

Ultimately, it will be key to understand where and how mis-splicing of mutant huntingtin occurs in humans and its contribution to pathology. Anne Young suggested examining the levels of exon 1 mRNA in frozen HD brain tissues. Moreover, she proposed using laser capture micro-dissection to assess local levels of exon 1 mRNA and correlate them with associated degrees of CAG somatic expansion. Housman pointed out, however, that most human tissues derive from individuals in the late stages of disease, at which point key brain regions are already too damaged to be analyzed. McDonald suggested examining less vulnerable parts of the brain to circumvent, at least to some extent, this problem. Moreover, DiFiglia noted that searching for the exon 1 fragment in a peripheral tissue might prove more fruitful. In particular, she suggested using tissue from testes which express high levels of huntingtin.

*What are the mechanisms underlying aberrant splicing?*

Participants were also curious about the mechanisms and regulation of the splicing process. Zeitlin said he would like to study the sequences in exon 1 that affect splicing. Moreover, Bob Hughes pointed out that there appears to be a discontinuous change in huntingtin mRNA secondary structure around 36 CAG repeats, and wondered if this matches the threshold at which aberrant fragments are observed. He added that computational modeling might provide insights into this question. Housman said the amount of aberrantly spliced fragment generated is clearly dependent on CAG length, but whether there's a specific length that marks a transition state remains unknown. He also noted the diversity of modeling approaches to determine RNA secondary structure and the difficulty of knowing which one will yield reliable results. McDonald suggested doing nuclease protection assays which can now be carried out in single cells, across the whole genome or transcriptome. Housman said he and his collaborators are currently working on this.

Identifying the splicing factors involved in generating the exon 1 fragment was also highlighted as an important next step. As noted in the January workshop, Bates and Housman used bioinformatics to examine exon 1 and identified a CAG or CAGCAA repeat as a binding site for the splicing factor SRSF6. RNA co-immunoprecipitation experiments using an antibody against SRSF6 pulled down mutant huntingtin 5' UTR and early intron 1 sequences, but not transcripts containing exon 2 sequences. SRSF6 regulates splicing and facilitates translation of partially spliced transcripts. In addition, SR proteins can displace the U1 snRNP, promoting polyadenylation from cryptic poly-A signals within introns. Thus, the increased association of SRSF6 with expanded CAG repeats could account for the production of the aberrantly spliced exon 1 transcript in a CAG repeat-dependent manner.

Bates said her team will be collaborating with another lab to investigate the effects of manipulating SRSF6. In addition to deepening the understanding of her findings, the experiments could give an indication of whether targeting the splicing machinery is of therapeutic interest. Diane Merry was enthusiastic about these experiments and pointed

out that her team has knocked down splicing factor SRSF2 and observed encouraging effects on a disease model that involves aberrant splicing. In addition, Hughes noted that exon skipping technology, an approach that uses antisense oligonucleotides to mask exons from being translated, is yielding encouraging results for the potential treatment of spinal muscular atrophy. Although this specific therapeutic strategy is not applicable for the treatment of HD, it suggests that the RNA processing machinery is druggable.

### **Post-translational modifications: an emerging source of potential therapeutic targets**

Participants also discussed new findings indicating that post-translational modifications are key determinants of mutant huntingtin toxicity. Updates on the roles and therapeutic options of targeting phosphorylation, acetylation, SUMOylation and neddylation were presented and discussed. The regulation of clearance through both proteasomes and autophagy emerged as a particularly promising avenue for therapeutic intervention.

#### Phosphorylation

The first 17 amino acids of huntingtin (N17) have been the focus of much attention recently because they are highly regulated, being the target of at least 11 post-translational modifications, some of which have significant effects on toxicity. Most strikingly, in 2009, William Yang and colleagues reported that mice expressing mutant huntingtin with amino acid replacements that mimic the phosphorylation of serines 13 and 16 are rescued from developing the motor and behavioral deficits associated with HD, as well as from developing mutant huntingtin aggregates and selective brain atrophy.

These results have been followed up with studies that reveal a complex picture of how N17 and its phosphorylation affect mutant huntingtin toxicity. At the HD2010 and HD2012 meetings, for example, Ray Truant described that phosphomimicry of serines 13 and 16 in isolated cells, shifts the aggregate population towards fibrillar forms (presumed to be less toxic), whereas kinase inhibition results in more globular aggregates. In addition, the phosphorylation status of N17 seems to be involved in determining the subcellular localization of mutant huntingtin, with increased levels of phosphorylated species localizing to the nucleus.

More recently, Yang observed that deleting the N17 domain of mutant huntingtin in BACHD mice results in a dramatic acceleration of nuclear aggregate formation and a very severe phenotype. As Yang described at the 2012 Boston Meeting, the animals expressing this deletion mutation showed HD-like symptoms earlier, including depression-like behavior and impaired rotarod performance. They also began suffering at an earlier age from progressive weight loss, progressive and selective forebrain atrophy, loss of DARPP-32 neurons, gliosis, and changes in striatal gene expression similar to those observed in human HD.

As described by Yang, his current hypothesis is that the N17 domain plays a key role in keeping the exon 1 fragment out of the nucleus. Moreover, at the 2012 Boston meeting, Yang suggested that early in HD, N17 may be protective, perhaps by carrying out its important roles in mediating and regulating normal huntingtin functions, including

selective autophagy (see below *More facets of the post-translational regulation of huntingtin clearance*). Eventually, however, this beneficial role might be overwhelmed by the disease process and N17 then becomes a contributor to toxicity, perhaps by accelerating aggregation, and/or interfering with mutant huntingtin clearance and localization. These mice also have a much more dramatic behavior resembling chorea in humans.

To follow up on his studies, Yang plans to sequence N17 in patients to search for variants that correlate with particular disease phenotypes. In addition, he is currently examining the activities of kinases that might regulate N17 activity and be of therapeutic relevance. Young added that it would be of interest to investigate whether mice expressing mutant huntingtin constructs with phosphomimicry of serines 13 and 16 generate aberrantly spliced exon 1 fragments. Although there is no known link between these two processes, Young noted that there might be some sort of feedback that affects the splicing reaction. McDonald pointed out that there is some anecdotal evidence suggesting mutant huntingtin affects splicing.

#### *Neddylation and ubiquitination*

Participants also discussed exciting new results indicating the involvement of post-translational modifications in huntingtin clearance. Marian DiFiglia, for example, described the recent identification of the negative regulator of ubiquitin-like protein 1 (NUB1) as a protein involved in enhancing mutant huntingtin degradation by proteasomes. NUB1's role in huntingtin clearance was suggested by its appearance in a genome-wide RNA interference screen for genes modifying mutant huntingtin abundance. As explained in a recent publication by James Palacino and colleagues, NUB1 reduces mutant huntingtin levels by enhancing polyubiquitination in collaboration with the ubiquitin-like protein NEDD8. As a potential approach to modulating NUB1 for treatment, the researchers tested the effects of interferon- $\beta$ , an inducer of NUB1. The treatment lowered mutant huntingtin and rescued neuronal toxicity.

#### *SUMOylation*

Huntingtin SUMOylation, a post-translational modification regulated by phosphorylation, also affects huntingtin clearance and can be modulated to enhance neuroprotection. Indeed, Leslie Thompson's team recently discovered that downregulation of PIAS1, an enzyme involved in the SUMOylation of huntingtin, is neuroprotective in a *Drosophila* model of HD. PIAS1 is a SUMO ligase that mediates the SUMO-1 and SUMO-2 modification of huntingtin. As described in a recent paper by Thompson and colleagues, SUMO-2 modification regulates accumulation of insoluble huntingtin in a manner that mimics proteasome inhibition and can be modulated by overexpression and acute knockdown of PIAS1. The article also notes that "the accumulation of SUMO-2-modified proteins in the insoluble fraction of HD postmortem striata implicates SUMO-2 modification in the age-related pathogenic accumulation of mutant HTT and other cellular proteins that occurs during HD progression."

Thompson noted that PIAS1 and SUMOylation are emerging as potential drug targets, not only for HD but for other neurodegenerative disorders, such as spinal bulbar muscular

atrophy (SBMA) and spinocerebellar ataxia (SCA) types 7 and 1. Diane Merry added that SUMOylation of the androgen receptor modulates its transcriptional activity and, in SBMA, is protective.

Thompson and her team are now working towards understanding more of the details of PIAS1 regulation and interaction with huntingtin. First, they plan to reproduce some of their key findings in BAC-HD mice and investigate the effects of downregulating or overexpressing PIAS1 in specific parts of the brain. PIAS1 is expressed in multiple tissues, but is particularly abundant in the brain. The team may also cross PIAS1 knockout mice with HD mice to assess whether the HD phenotype is ameliorated as expected. Thompson is also interested in assessing whether searches for modifiers of HD pick up PIAS1.

Participants asked about the baseline levels of PIAS1 and huntingtin SUMOylation in wildtype and HD models to assess the context in which a putative therapy that modulates PIAS1's activity would be operating. Thompson said there doesn't seem to be a significant difference between wildtype levels of PIAS1 and those found in HD, although R6/2 mice may have a small degree of dysregulation, human levels have yet to be measured. She also noted that both wildtype and mutant huntingtin are SUMOylated. Ai Yamamoto asked how huntingtin SUMOylation changes during development. Thompson noted that this is hard to assess because SUMOylation is very rapid and transient.

Steffan underscored the importance of considering timing when manipulating SUMOylation given that maintaining the right cellular balance of SUMO proteins is critical for cellular health. Moreover, Jang-Ho Cha added that it will be useful to know which other proteins, in addition to huntingtin, would be affected by the modulation of SUMOylation. To do so, he proposed examining the SUMO-ome in SUMO-1 and SUMO-2 knockout mice. Thompson said her team had written a grant to do this, in addition to assessing global transcription levels, but unfortunately, it did not get funded.

#### *More facets of the post-translational regulation of huntingtin clearance*

Yet another clearance pathway that may be regulated at the post-translational level and also be a target for HD therapeutic intervention is autophagy. Joan Steffan noted that huntingtin likely plays a role in selective macroautophagy, a process which is involved in huntingtin's own lysosomal degradation. She pointed out that huntingtin has similarities to several proteins required for selective autophagy in yeast. In addition, data from Ana Maria Cuervo's lab indicate that huntingtin may have a normal role in macroautophagy, possibly in cargo recognition. Steffan further explained that the phosphorylation and acetylation of residues within huntingtin's N-terminus may participate in the regulation of huntingtin's role in autophagy.

As pointed out by several participants, a key factor to consider will be the timing of potential interventions. Hughes noted that studies in *C. elegans*, for example, show that cells in younger animals have high levels of disaggregation activity, whereas later in life, aggregation activity increases. The observations suggest that strategies to deal with toxic proteins may change with age and/or disease stage. Hughes added that as one mechanism

becomes overburdened, another one might take over. McDonald wondered if studying *C. elegans* in the dauer stage, a long-lived larval stage that animals enter under harsh conditions and which has helped reveal longevity pathways, might be informative. In particular, it could help dissect which changes in clearance are due to aging and which ones are due to disease stage. As noted by Steffan, such experiments could also help reveal links to metabolism.

Addressing huntingtin clearance in particular, Steffan has been studying huntingtin's phosphorylation and acetylation patterns in wildtype and mutant mice over time and observed that these modifications accumulate with age, as do huntingtin clearance intermediates. She proposed that pharmacologic activation of selective macroautophagy mediated by huntingtin may help slow neurodegeneration early in disease progression.

All agreed that more work is needed to better understand how age and disease stage might affect the outcomes of modulating huntingtin's various post-translational modifications. In addition, as pointed out by DiFiglia and Merry, understanding the upstream and downstream pathways associated with the modifications, and their own timing and feed forward effects, will be of interest and may reveal more therapeutic candidates. Steffan agreed and noted that investigations in this area are ongoing. For example, she and her co-workers have now established that the kinase IKK plays a key role in mediating phosphorylation, as well as acetylation and poly-SUMOylation in huntingtin's amino terminus. In addition, mutant huntingtin activates the IKK kinase complex which affects transcription, and which may also have direct effects on huntingtin post-translational modification.

Despite these complexities, Steffan emphasized the advantage that HD has over other neurodegenerative disorders regarding the timing of therapeutic interventions. Because HD can be identified and treated pre-symptomatically, it offers more treatment options and the possibility of blocking pathological processes before they have caused too much damage.

### **Additional sources of therapeutic targets**

#### Neuroinflammation

Participants also touched upon other mechanisms of pathology that are of emerging interest. Neuroinflammation has long been known to play a role in the damage observed in several neurodegenerative disorders, including HD. But effective therapies based on dampening this response have been difficult to develop. Participants were encouraged, however, by Alex Kazantsev's recent identification of a novel lead compound, MIND4, that downregulates neuroinflammation. As explained by Anne Young, MIND4 potently represses the expression of inflammatory markers in activated microglial cells through activation of the NRF2/KEAP1/ARE pathway. In particular, MIND4 dissociates KEAP1 from NRF2, allowing it to go into the nucleus. Through this mechanism, MIND4 has been shown to be protective in primary neurons, neuronal slice cultures and a *Drosophila* model of HD. A group of researchers, including DiFiglia and Thompson, are now expanding these studies to include mouse models of HD. So far, they have found that

MIND4 treatment significantly reduces levels of TNF-alpha, an adipokine involved in systemic inflammation, in the cortex of symptomatic HD mice.

The compound is particularly promising given that, as noted by Young, it is approximately ten times more potent and more selective than dimethyl fumarate, a drug with immunomodulatory properties developed by Biogen and recently approved by the FDA to treat multiple sclerosis. MIND4 readily crosses the blood-brain barrier and appears to be protective, not only in HD models, but in Parkinson's disease and possibly amyotrophic lateral sclerosis. Kurt Fischbeck added that its effects on Kennedy's disease are also being examined. Young stressed the importance of obtaining funding for streamlining the development of this promising candidate.

#### *HD-associated disruption in metal homeostasis*

Another aspect of HD pathology that was discussed at the workshop was the role copper may play in exacerbating mutant huntingtin toxicity. Ira Shoulson described a new study by Bing Zhou and colleagues showing that copper appears to alter the expression of genes involved in copper metabolism and accelerates HD progression in a *Drosophila* model of the disease. Conversely, reducing copper decreases levels of huntingtin oligomers and aggregates. The report presents some evidence for a direct interaction between huntingtin and copper, but the mechanistic underpinnings of the findings are still uncertain.

Shoulson added that other studies in Alzheimer's disease and, more recently in HD, also point to copper modulation as a target for therapeutic intervention. The studies involve testing of a small molecule, the copper/zinc ionophore PBT2, which has been shown to improve cognition in mouse models of Alzheimer's disease (AD), as well as in a small phase II clinical trial with individuals suffering from AD. There is some indication that the drug also improves motor function and reduces neurodegeneration in animal models of HD. Based on these findings, a clinical trial is underway to evaluate the safety and tolerability of PBT2 and establish a dose-response curve in individuals with early to mid-stage HD. As explained by Shoulson, this phase II study involves 108 individuals over a period of 6 months.

Participants also pointed out that the PBT2 studies do not include an assay to determine whether the intended target is being engaged or not. As noted by Jang-Ho Cha, if no effect is detected, it will be impossible to know whether it was due to a fundamental problem with the drug's mode of action or to insufficient target engagement. Shoulson agreed and recounted a situation in which a copper-sequestering agent had been tested to treat Wilson's disease, a genetic disorder in which copper accumulates in tissues, but failed because it was unable to cross the blood-brain barrier.

Despite this shortcoming, Cha noted that the size and scope of the trial make it more affordable than others and, consequently, of potential interest to companies like Merck. He considered that the approximately \$10 million dollars such a study costs, would be a low price to pay for a proof-of-concept, phase II trial of that size. However, Shoulson cautioned that the results from the PBT2 study might not be as tidy as those obtained

from comparable trials, such as those carried out to test the anti-choreic medication tetrabenazine, for example. Much was known about tetrabenazine beforehand, whereas there are many uncertainties still surrounding the therapeutic potential of PBT2.

### More targets on the horizon

Several ongoing projects promise to provide yet additional targets for therapeutic intervention. William Yang, for example, is conducting high throughput screens to elucidate the huntingtin “interactome.” In addition, Bob Hughes is performing large scale, global RNAi screens to “map out the landscape of molecules that influence huntingtin.” Hughes is also interested in examining molecules that control cell growth and shape because of huntingtin’s apparent role in neurite growth, originally suggested by anatomical observations from Marian DiFiglia’s lab.

## **Gene Silencing**

As described in previous workshops and meetings, gene silencing is one of the most promising and advanced candidate therapies in the HD pipeline. At this workshop, participants discussed new options for the delivery of silencing agents, timing of treatments, selection of sequence targets, and strategies to track target engagement.

### Delivery

As noted by several participants, there are still a few challenges that need to be resolved to enable the safe and effective delivery of silencing agents in HD. As noted by DiFiglia, the persistence of siRNA agents delivered via adeno-associated viruses (AAV) is cause for some concern. As described in previous workshops, the inability to control the expression of these vectors has been problematic because treatments are essentially irreversible—they cannot be switched off if they prove harmful. Furthermore, treatments cannot be tailored to match symptom severity. To address these issues, several groups are working on generating conditional siRNA constructs. Ultimately, said DiFiglia, the goal is to have an implanted source of silencing agents that can be regulated peripherally.

Another issue that needs to be addressed is developing a better understanding of AAV receptivity amongst different neuronal types. Although much is already known about the selective tropisms of AAV serotypes for different cell types, such as neurons versus glia, more fine-grained information is needed regarding the receptivity of specific neuronal types. DiFiglia noted that she and Aronin are currently working on this. Young wondered if some of these delivery questions in humans might be amenable to investigation by running controlled tests in two-stage surgeries used to treat medically refractory epilepsy. The first surgery serves to map the brain region that is affected and determine which area will be removed in the second surgery. If an experimental AAV construct could be administered to the affected brain region in the first surgery, then the fate of the vector could be examined after its removal in the second surgery.

One of the major challenges for moving the gene silencing approach to the clinic, however, is finding a way to distribute silencing molecules in large brains. Because HD is a disease that affects neuronal function throughout the brain, it will be necessary to



distribute silencing agents widely or at least to the most vulnerable regions which are difficult to cover, the striatum lying deep within the brain, and the cortex which extends over the entire surface of the brain. To address this issue, DiFiglia and Neil Aronin are testing the use of exosomes as carriers in sheep which, like humans, have large brains. Exosomes are nano-sized vesicles produced naturally by many cell types loaded with miRNAs that influence various physiological and developmental processes. Exosomes have tetraspanin surface domains which confer selectivity for cargo delivery to specific targets. Recent recruitment of these nanocarriers for delivery of nucleic acids in experimental systems is proving very powerful and researchers in several fields are currently exploring their potential for clinical use.

Other approaches to the delivery problem were also discussed. David Housman, for example, noted that the biotech company Isis is using a proprietary approach developed by Roche that involves targeting the transferrin receptor with a low-affinity antibody. The technique is expected to allow the delivery of antisense oligonucleotides (ASOs) to the brain via systemic administration.

An particularly intriguing new approach for delivering silencing molecules was presented by Juan Sanchez-Ramos. Searching for a non-invasive way to deliver silencing agents, Sanchez-Ramos tested whether nanoparticles containing manganese could be used to deliver nucleic acids through the nose. Manganese is actively taken up through the nose and into the olfactory bulb where it is transynaptically transported to other neurons throughout the brain. Furthermore, Sanchez-Ramos explained that, although manganese can cause anosmia when delivered through the nose and can be reversibly toxic in certain brain regions, such as the globus pallidus, in small doses, it appears to be essentially harmless in healthy individuals. Indeed, manganese has been approved by the FDA as a contrast agent for magnetic resonance imaging.

To test his idea, Sanchez-Ramos initially generated 100 nm particles containing an RFP construct coated with manganese. The particles were then given to mice nasally. When RFP production was monitored a few days later, the researchers found that the construct expressed at high levels in various brain regions, including the basal ganglia and cortex. To test if a silencing agent would also work, Sanchez-Ramos treated transgenic mice expressing GFP in all tissues with GFP siRNA. A 30 percent reduction in GFP expression in the cortex was observed using real-time PCR. The researchers also discovered, from their work in cell culture, that manganese bolsters transfection efficiency without interfering with silencing. Now Sanchez-Ramos hopes to use atomic magnetic spectroscopy to track manganese in the brain and correlate it with GFP levels of expression.

Participants asked about the mechanism underlying manganese transport. The issue is complicated because Mn (2+) and Mn (3+) are transported differently and the mechanisms can vary between cells. According to a recent article by Gunter and colleagues, Mn(2+) is transported into cells via a number of mechanisms, while Mn(3+) is believed to be transported similarly to iron via the transferrin system. Cellular uptake is

therefore determined by the activity of the mechanisms transporting manganese into each type of cell and by the amounts of Mn(2+), Mn(3+) and their complexes.

Participants also had questions about the potential effects of manganese in individuals suffering from HD. Young pointed out that metal homeostasis is altered in HD, as observed by Diana Rosas. Moreover, Ira Shoulson noted the recent publication of a paper showing that copper appears to bind to mutant huntingtin, and elevated levels of this metal accelerate HD progression in *Drosophila* (see below “HD-associated disruption in metal homeostasis”). Sanchez-Ramos added that elevated manganese levels can alter iron transport, possibly through its interaction with the transferrin transport system. Furthermore, anosmia is a potential side-effect of intranasal manganese administration and Thompson noted that this same problem has been associated with HD. Sanchez-Ramos considered that because manganese appears to be harmless in healthy individuals, but might aggravate some HD problems, it may be best to test the manganese delivery system in individuals in the early stages of disease.

Participants agreed that Sanchez-Ramos’s findings are encouraging and offered suggestions on how to move forward. Sanchez-Ramos said that one of his top priorities is to investigate dosage to establish the range at which the carrier is safe and most effective. Marie-Francoise Chesselet offered to connect Sanchez-Ramos with an expert in manganese toxicity at UC Santa Cruz who has very reliable methods for measuring this metal *in vivo*. In addition, Hughes suggested looking into generating high affinity aptamers that bind to the manganese transporters to potentially replace manganese as a carrier with a more specific and potentially safer molecule.

Sanchez-Ramos also noted that he plans to begin testing the delivery of huntingtin silencers in induced pluripotent stem (iPS) cells derived from HD fibroblasts and then running experiments in mice. Leslie Thompson offered to help with these efforts, pointing out her lab’s experience working with both iPS cells and nanoparticles. Moreover, Chesselet offered to share a protocol to administer compounds nasally in mice without the need for anesthesia. She said her team has achieved brain-wide distribution of a small peptide in a mouse model of Parkinson’s disease via nasal delivery without using anesthesia.

Participants also discussed the possibilities for using manganese to deliver other compounds, in addition to gene silencers. Sanchez-Ramos said that his patent covers the delivery of a wide range of large molecules, including peptides, proteins and nucleic acids. He noted that his group is considering many applications for the technique, including for example, the delivery of the neurotrophic factor BDNF to the brains of animal models of Parkinson’s disease. Kurt Fischbeck wondered if intrabodies could be delivered and Sanchez-Ramos said it should be possible because the nanoparticles are internalized into the cell cytoplasm. Moreover, Shoulson pointed out the potential utility of the technique for palliative treatments of HD, in addition to its use as a carrier to deliver huntingtin gene silencers. For example, Shoulson said an oxytocin analog delivered nasally is currently being tested to treat irritability and aggression in other disorders.

Jang-Ho Cha offered to help Sanchez-Ramos plan for future funding and development of his idea. So far, Sanchez-Ramos said he has received support for some of this work from a family in Miami, but he needs to find additional sources of funding. He said he approached the CHDI, but they would not consider funding until he obtains data showing the specific silencing of huntingtin expression. Cha opined it might be best for Sanchez-Ramos to form his own company and do at least some of the development outside of the grant system.

#### Timing and magnitude

Another important issue discussed at the workshop was the optimization of the timing and magnitude of huntingtin reduction mediated by gene silencing therapies. Scott Zeitlin pointed out that huntingtin is a multi-functional scaffold protein involved in many cellular processes, including anterograde and retrograde axonal transport, membrane trafficking, gene expression and RNA regulation. Consequently, it will be important to determine how therapies that reduce huntingtin levels chronically will be tolerated, and how this will be affected by the age and stage of disease at which the therapy is initiated.

To investigate this issue, Zeitlin is generating mouse lines expressing either both mutant and wildtype huntingtin, or only mutant huntingtin, under the control of a lac repressor system. In this manner, he hopes to recreate conditions similar to those expected from either non-allelic specific, or allele-specific, silencing of mutant huntingtin and examine the effects of reducing huntingtin levels at different ages and stages of disease. Zeitlin noted that the repression levels he has achieved are between 30 and 40 percent, levels which are comparable to those expected from the first generation of huntingtin gene silencers. So far, Zeitlin has only examined 6 month-old mice and observed that reducing huntingtin expression at three months of age postpones the appearance of inclusions by approximately three months. Zeitlin is most interested in examining mice treated later in the disease process, however, to more closely reflect the expected timing of silencing therapies in initial clinical trials.

#### Tracking huntingtin reduction

As stated previously, using biomarkers to track target engagement is key to conducting informative clinical trials. Shoulson noted that measuring mutant huntingtin levels peripherally may offer an effective, minimally invasive way to monitor target engagement in gene silencing therapies. Shoulson explained that Steve Hersch and colleagues recently reported measuring the levels of mutant huntingtin and total huntingtin in blood leukocytes from Prospective Huntington At-Risk Observational Study (PHAROS) subjects. Using a homogeneous time-resolved fluorescence (HTRF) assay to monitor huntingtin in close to 400 samples, the group established that the assay can effectively measure mutant huntingtin in multicenter sample sets and conclude that it may be useful in trials of therapies targeting huntingtin. Shoulson added that using forearm perfusion techniques to obtain multiple samples over time, one could analyze the kinetics of silencers' effects on huntingtin levels.

Hughes cautioned, however, that peripheral huntingtin levels may not accurately reflect huntingtin levels in the brain. For example, false negatives could emerge if a silencing approach works in the brain without affecting systemic huntingtin levels. Shoulson acknowledged this possibility, but noted that the potential of peripheral biomarkers has been generally underappreciated and should be investigated more thoroughly. There are a number of tissues, such as blood, muscle, islets of Langerhans and skin that could prove useful in the study of a number of therapeutic candidates. Indeed, Ai Yamamoto noted her team is working on the identification of several new peripheral biomarkers.

#### *Selection of silencing agents' sequence*

As discussed at the January workshop, another challenge facing gene silencing approaches is that the majority of huntingtin sequences being targeted with current silencers reside downstream of huntingtin exon 1. This could be problematic in light of Bates's and Housman's discovery of the aberrantly spliced exon 1 fragment mentioned above (see "Aberrant splicing of mutant huntingtin mRNA"). In the early days of the development of siRNAs and ASOs against huntingtin, several exon 1 silencers were found to be toxic because of off-target effects and efforts were subsequently focused on sequences downstream. In addition, several allele-specific approaches rely on targeting single-nucleotide polymorphisms (SNPs) in the 3' UTR of huntingtin mRNA.

Nevertheless, developing new silencers that target both full-length huntingtin and the mis-spliced fragment might be possible and desirable. As noted by Marina Chicurel, Beverly Davidson mentioned at the January workshop that it is likely there are alternative exon 1 sequences that do not have off-target effects and could be useful targets. Housman added that his team has evidence for effective target sequences in the 5' UTR. Moreover, Kurt Fischbeck noted that it might be helpful to work with a reporter construct to systematically test sequences along the huntingtin transcript's length, as was done in the search for silencers to treat spinal muscular atrophy.

Participants also wondered about the effects of the Isis anti-huntingtin ASO on the mis-spliced exon 1 fragment. Working with Roche, Isis is ramping up to start a clinical trial but, as noted by Housman, the sequence of their oligonucleotide has not been disclosed yet. An investigational new drug (IND) application will be filed soon and this document should contain the information. As noted by Hughes, the Isis ASO is not expected to downregulate the exon 1 fragment because its target is, as that of most silencers under development, downstream of exon 1.

Nevertheless, Bates and Chesselet are interested in examining its effects because there are still many open questions about huntingtin mRNA processing. For example, as pointed out by Chicurel, some pre-mRNAs are exported to dendrites where calcium signaling activates their splicing. As noted earlier, huntingtin mRNA has been found in dendrites, so it is possible that some of its processing occurs at this subcellular location. If so, silencers that target sequences downstream of exon 1 might have the opportunity of downregulating exon 1 fragment production. Chesselet noted that her team used the Isis ASO in animal experiments funded by the CHDI, and had requested that she send Bates these tissues to examine for the presence of the aberrantly spliced fragment.

Unfortunately, this was difficult to do because of restrictions in the contract between Isis, CHDI and the Chesselet lab. This analysis will now be performed by the Chesselet lab in-house.

### Genome editing

Although only briefly, participants also discussed an intriguing alternative to gene silencing known as genome editing—a technique in which DNA is inserted, replaced or removed from a genome using engineered nucleases. There are currently several families of nucleases being used for this purpose, including the newly recruited CRISPR system which appears to be very powerful. The CRISPR (clustered regularly interspaced short palindromic repeats) system was first identified in prokaryotes as a defense system against phage infection which relies on sequence-specific cleavage of foreign nucleic acids. Short segments of the foreign DNA are incorporated into the host genome between CRISPR repeats and serve as a memory of past exposures. These segments are then used to produce short RNAs that direct the degradation of foreign nucleic acids in a process similar to eukaryotic RNA interference. In 2012, George Church and colleagues showed that the system could be engineered to function with custom guide RNA (gRNA) in human cells to allow genomic editing.

As described by Housman, the technique is extremely efficient, cheap and easy to manipulate. And it has proved effective in correcting mutations in a variety of systems, including human iPS cells. However, Housman noted that off-target effects have been observed which can lead to non-specific mutations elsewhere in the genome. Several groups are working to address this issue. For example, Church's group recently generated a series of endonuclease Cas9 mutants that are expected to reduce non-specific cutting. Other groups are working to recruit and enhance the editing capabilities of other nucleases, such as zinc finger nucleases and transcription activator-like effector nucleases (TALENs). For example, as noted by Housman, the biotech company Sangamo has created zinc finger nucleases which enable gene-editing in cells.

### **Use of tissue collections and databases**

As noted by N. Wexler and Young, with all these new findings, powerful technologies and intriguing questions to be answered, it is time to think about how best to use the valuable collections of HD tissues and databases that the Hereditary Disease Foundation and others have put together and maintained over the years.

The Venezuelan kindreds, for example, comprise over 18,000 individuals, 83 unique kindreds, and span 10 generations. As described by N. Wexler, the prospective database of these families contains clinical and genetic information, spanning 23 years and including over 2,500 individuals who were examined almost every year. In addition, there are over 4,000 immortalized lymphocyte lines derived from these families. Also, noted Jacqueline Jackson, the *National Research Roster for Huntington Disease Patients and Families* at Indiana University manages a DNA bank with approximately 4,000 samples from HD families in the U.S., including samples from a large American family in Iowa. As explained by Jackson, the samples belong to the families who pay \$100 for

storage. N. Wexler considered this an excellent way to build a repository, particularly because participants are not required to be genetically tested in order to store their samples.

Shoulson added that several observational HD studies are generating additional tissue collections and large databases of great value. For example, the COHORT study, organized by the Huntington Study Group, enrolled over 2,500 individuals from HD families in the U.S., Canada and Australia. Blood samples, results from neurological tests, and medical histories have been collected annually for 5 years. COHORT has now merged with REGISTRY, a similar study based primarily in Europe with over 10,000 participants, to create ENROLL-HD. The primary objective of ENROLL-HD is to develop a comprehensive repository of prospective and systematically collected clinical research data (demography, clinical features, family history, genetic characteristics) and biological specimens (blood) from individuals with manifest HD, unaffected individuals known to carry the HD mutation or at risk of carrying the HD mutation, and control participants. The study includes over 200 sites in roughly 30 countries.

#### *New ideas to use databases and tissues*

Several participants said they would like to search for correlations and patterns in the databases. As an example of the value of such analyses, Shoulson noted that analysis of the COHORT database recently revealed heart rate as the single feature that clearly differentiated between individuals having more than 37 CAG repeats in a huntingtin allele and those having less. Consistent with this finding, another study found that increased heart rate was a strong predictor of disease in both pre-manifest and manifest stages of HD. Shoulson added that anxiety or increased movements did not seem to underlie the difference and no significant differences in blood pressure were observed between the two groups. Participants mentioned several HD-associated disruptions that could be relevant to the findings, including metabolic alterations, disruptions in vagal innervation, and altered body temperatures. Chesselet added that electrocardiograms are currently being used to monitor Parkinson's disease and Young said Diana Rosas is beginning to search for HD-associated alterations in electrocardiograms.

Young suggested using the databases to look for a link between HD progression and CAG repeat length. She noted that Jean-Paul Vonsattel had observed a correlation between brain pathology and repeat length, but a more comprehensive study would be of interest. She suggested focusing on progression in the linear phase of the disease, excluding individuals who are moribund for years and individuals with the juvenile form of HD. Cha added that two analyses could be performed, one including, and one excluding, juveniles. The COHORT study failed to reveal a link between progression and repeat length, noted Shoulson, but nevertheless, he supported performing the suggested analyses. A more sophisticated analysis, including data over a longer time period, said Shoulson, might reveal a previously undetected relationship.

Another suggestion for the use of the rich databases was put forth by Jang-Ho Cha who noted that the smart marriage of motor data sets and associated neuropsychological data might reveal distinct types of HD and/or distinct patterns of disease progression. These

analyses could help stratify patients for treatments. For example, as noted by Young, specific groups of individuals might benefit from the silencing of huntingtin in different brain regions. Also, such analyses might help identify the cleanest biomarkers to track progression in different groups of individuals, noted Cha, as well as help design a composite biomarker that could serve as a universal biomarker for HD, as is currently being done for Alzheimer's disease.

Cha also noted that this type of analysis could help identify groups that might be better suited for particular drug trials. For example, to test a drug that is expected to slow disease progression, it would be helpful to enroll individuals whose disease progresses fast. Hughes added that the proposed stratification could also bolster the power of studies searching for modifier genes (see "Modifier Searches: Challenges"). It is likely that different types of HD will be associated with different modifier genes and separating these groups out should help make associations easier to identify. As noted by Young, data from Richard Faull's team indicate that there are indeed different flavors of HD, with some individuals showing more motor pathology and symptoms, while others have more mood alterations.

Participants agreed that using bioinformatics to analyze the large HD datasets is likely to yield many types of valuable information. DiFiglia suggested hiring a bioinformatics core, which could be done right away without incurring a very large expense. Hughes agreed and added that getting the database resources ready for analysis should be a top priority for the Hereditary Disease Foundation.

Participants also discussed several ideas for using the HD tissue collections to advance HD research. For example, Gill Bates proposed examining fibroblast lines derived from individuals at different stages of disease. It is not possible to establish fibroblasts from the R6/2 mouse model at late stage of disease and therefore phenotypes may be present in fibroblast lines that are related to the stage of the disease at which the line was established. It would be extremely useful to have a bank of fibroblast lines that are stratified for CAG repeat length and stage of disease at which they were collected. For example, the ability to mount a heat shock response declines with disease progression in HD mouse models. Bates was not able to investigate whether this might also be applicable to HD patient fibroblasts as many of the HD lines that she has were collected from presymptomatic individuals.

In addition, Marian DiFiglia, who has been investigating the role of NADPH oxidase (NOX) activity in HD, said she was interested in examining NOX2 activity in the Venezuela cell lines. At the January workshop, DiFiglia described how reducing NOX2 activity in HD mice normalizes the levels of reactive oxygen species in the brain and significantly improves survival. The findings are of interest because they point to an early alteration in HD that could act as a trigger for other, later-stage changes in neuronal function.

Moreover, Leslie Thompson suggested using the Venezuelan cell lines to increase the number of homozygotes in the HD iPS cell collection. Currently, the collection has only

one or two homozygote lines and adding a few more with solid pedigree data, would be of great value. In addition, Thompson noted that a cell line from one or more individuals with either an unexpectedly late or early age of onset (outliers) would be useful for ongoing modifier studies using iPS cells (see “Modifier Searches”). McDonald agreed and added that the Venezuela tissues could be used to genetically examine the whole gamut of phenotypic extremes—not only outliers in age of onset, but in progression rate, mood disruptions, motor alterations, etc.—to perform focused modifier searches.

Furthermore, Yamamoto said she was interested in examining circulating insulin and glucose levels, and assessing HD-associated alterations in vesicle trafficking. Young noted that the Venezuela samples do not include blood, but the PHAROS collection might prove helpful. In this study, 1001 adults at 50-50 risk for HD agreed to provide longitudinal clinical data and a blood sample. Hughes pointed out that immune function might also be interesting to assess in the Venezuelan tissue samples given that this population has probably been exposed to high levels of infectious agents.

#### *Challenges and ideas for future improvements*

Participants also discussed some of the current limitations and problems associated with the tissue collections and databases, as well as ways to address them. As noted by Simone Roberts, for example, the Venezuelan pedigree data is not in an electronic format. Part of the problem is the size and complexity of the kindreds which make them difficult to handle with conventional pedigree mapping software.

But even if the pedigree data were in an electronic format that was user-friendly and easy to share, it would still be difficult to make it widely accessible to the research community because of the risk of violating participants’ privacy. Shoulson explained that even when individuals are de-identified, pedigree structures can be used to determine subjects’ identities. McDonald added that Columbia University in particular has a very strict Institutional Review Board (IRB). Sometimes, noted Roberts, even the ages of individual subjects must be removed from datasets to be shared.

Maintaining subject privacy while maximizing research potential is a complicated issue that affects resources for biomedical research beyond the HD databases. For example, N. Wexler pointed out that the catalog for the Corriell Cell Repositories is including much less data than it once did because of privacy issues. McDonald and Thompson noted that, from a research perspective, this reduction is unfortunate.

A related issue which affects, not only privacy limitations, but other facets of tissue and data usage, is the wording of subject consent forms. As explained by McDonald, the use of samples, funding of research projects, as well as uploading of data into public databases, are all dependent on the use and appropriate wording of consent forms. For example, McDonald noted that she was once unable to obtain samples from a brain bank holding tissues from people who had explicitly said they wanted to donate their brains for research because the tissues were not formally consented.



Leslie Weiner added that, in some instances, consents are limited to a particular project, which is also problematic. For example, Chesselet described a case in which a cell line consented for diabetes research turned out to be very useful for a neurological application, but researchers were limited to using the cells for diabetes only. Chesselet noted that different universities have different guidelines for writing consent forms but, within each framework, it is best to describe the intended uses of samples as broadly as possible. Also, as noted by Shoulson, consent forms should include a statement enabling researchers to contact participants with follow-up questions. It is possible to re-consent samples, as noted by Weiner, but it is expensive to do so.

Participants also discussed several of the features that are desirable in a database. McDonald pointed to the COHORT database as a good model—it is set up so it's easy to share (available on a CD by emailing Ray.Dorsey@chet.rochester.edu), it has a good data dictionary, and family information is cleanly separated from other data to protect subjects' privacy. McDonald added that data dictionaries are not only useful for finding desired measurements, but for triggering new ideas. She suggested creating such a dictionary for the Venezuelan datasets.

Finding ways to link the various databases, including databases from other diseases or from participants' earlier medical records, was also discussed. Shoulson suggested organizing a bioinformatics workshop to discuss how to harvest data, while preserving subjects' privacy. Shoulson added that Arthur Toga and Neil Buckholtz could provide advice on organizing and implementing systems to share data and conduct bioinformatics analyses, given that they have led efforts to create sophisticated databases and repositories for Alzheimer's disease and Parkinson's disease.

### **In search of modifiers of HD**

For several years, researchers have sought to deepen our understanding of HD and identify new therapeutic targets by searching for modifier genes of HD in humans. Several studies have been performed resulting in the identification of approximately 50 candidate modifier genes. Yet, as noted by McDonald, none of these candidates has been replicated and, indeed, most have been shown to be false positives. As Cha wondered, do HD gene modifiers actually exist and, if so, how many might one expect to find?

#### *Challenges*

McDonald answered Cha's questions by noting that the most likely reasons for these failures are technical rather than biological. McDonald suspects HD modifiers exist and is not surprised they have not been identified yet because many of the studies to date have suffered from one or several technical problems. For example, some studies have used statistical models to analyze associations that do not fit the distribution of variants in the data (phenotype and CAG length, for example). Also, some studies suffer from population stratification, the presence of a systematic difference in allele frequencies between subpopulations in the study group due to ancestry. If the stratification is not corrected for, the genetic markers reveal ancestral associations, rather than associations relevant to the disease.

Another significant problem, noted McDonald, is that many studies have failed to precisely define the phenotype being used to find associations. Datasets that lack quantitative, reproducible measurements of phenotypes can compromise the validity of modifier searches as well as interfere with the confirmation and validation of identified candidates. Establishing standardized, robust phenotypic measures, however, can be difficult. For example, Shoulson and McDonald recounted the challenges they encountered defining HD motor onset. As explained by Shoulson, onset depends on detection, which can vary widely depending on the patient, physician, and detection methodology. Even for diseases marked by an acute damaging event, noted Weiner, onset can be difficult to establish. Stroke victims, for example, sometimes describe sensations of numbness perceived years before the reported ischemic event.

In the case of HD, the COHORT researchers realized they would have to define onset parameters more clearly after talking with McDonald and others. A forum was set up to decide how to modify data collection and organization. Shoulson noted that the process was challenging. Lesley Jones has worked hard at implementing the suggestions emerging from the forum, including correlating onset data from physicians, subjects and family assessments for the EHDN REGISTRY samples.

Weiner stressed the importance of setting up standards at the beginning of studies and McDonald emphasized the value of collecting data on quantifiable phenotypic features, as opposed to Gestalt assessments of the HD phenotype. Gustavo Rey said he hoped to improve and simplify cognitive neuropsychiatric measurements. In addition, Hughes pointed out that the recently developed assay to measure mutant huntingtin levels in blood might also provide a quantitative readout of potential value for modifier searches. Mutant huntingtin levels in blood are very steady, noted Shoulson, but at the time of phenoconversion the levels seem to dip. The reason for this reduction remains unclear (perhaps movement of mutant huntingtin into inclusions, noted Shoulson), but lends support to the idea of this measurement being of potential value beyond its use as an indicator for target engagement for gene silencer studies.

Another complicating factor in the search for disease modifiers is that some modifiers may affect only a subset of phenotypic features. Indeed, Chesselet pointed out that apolipoprotein E4, a well known modifier of Alzheimer's disease, was initially thought to have no effect on Parkinson's disease. However, more careful studies, examining individual components of the Parkinson's phenotype, have revealed that ApoE4 is, in fact, a modifier of certain cognitive deficits associated with the disease.

Also, as mentioned above, there might be different flavors of HD which are affected by different modifier genes. McDonald opined that defining and dissecting the HD phenotype as precisely as possible and then identifying individuals with extreme expressions of different phenotypic facets would be useful before engaging in a large genetic study.

The somatic expansion of huntingtin CAG repeats in the brain is also a complicating factor, noted Zietlin. Expansion rates in HD vary between organs, but the greatest instability is observed in the brain and correlates with neuropathology. This micro-chimerism could affect attempts at correlating genomic data obtained from blood cells, or even brain tissues, depending on the area sampled, with phenotypic features. Also, noted Zeitlin, a recent connection has been made between transposon activity in the brain and both age- and disease-related neurodegeneration. It is still unclear whether transposons are a cause or an effect of aging-related brain defects, but in either scenario, they could complicate the interpretation of studies aimed at correlating genomic variants with phenotypic attributes.

### *Successes*

Despite these complications, some headway has been made in the search for HD modifiers in humans. In a paper published in *Genetic Epidemiology* in 2008, a team of researchers led by David Housman reported the identification of several candidate loci. The paper explains that the age of onset of HD is inversely correlated with the CAG length in the huntingtin gene, with the repeat length accounting for 70% of the variability in HD age of onset. However, for most individuals worldwide the repeat length accounts for much less of the variability in age of onset. Ninety percent of individuals have expanded CAG repeats between 40 and 50. For these people, the size of their repeat length only determines 44% of the variability in their age of onset. The rest of the variability is attributable to genes other than the huntingtin gene (40 percent) and environmental factors (60 percent).

Based on these considerations, Housman and colleagues analyzed the large Venezuelan kindreds in which the HD gene was originally identified. As stated in the abstract, these kindreds offer greater analytic power than standard sib-pair designs and the team developed novel pedigree-member selection procedures to further enhance and maximize this power. The researchers used a 5,858-single-nucleotide-polymorphism marker panel to perform a genomewide linkage analysis. They discovered two novel loci on chromosome 2: chromosome 2p25 (LOD=4.29) and 2q35 (LOD=3.39) which may contain genes that modify age of onset. A third linkage peak on chromosome 6q22 (LOD=2.48) was also found which may confirm the most promising locus from a previous genome scan. Two other candidate loci on chromosome 5 (LOD=3.31 at 5p14 and LOD=3.14 at 5q32) were described as “suggestive”. As noted by the researchers, these regions harbor candidate genes that are potential HD modifier genes.

Other studies have made progress by eliminating possible modifiers of HD. For example, as noted by McDonald, a study demonstrating that the allele with the shortest CAG repeat length is not a modifier of disease (at least in a Northern European population), helped establish that HD is a truly dominant genetic disorder. In addition, McDonald noted that Jong-Min Lee and Gusella showed that, at least for one particular haplotype, the haplotype itself is not a modifier of motor onset (it will be of interest to see if other haplotypes, such as those from Venezuela, yield the same result). Moreover, Lee and Gusella have also shown recently that variations in regulatory sequences near the huntingtin gene loci do not seem to influence HD pathogenesis. This finding should

facilitate the analysis of genome-wide association scans because it suggests that the full effect of the huntingtin locus can be attributed to the expanded CAG repeat, without significant contributions from neighboring genetic variations.

### *Looking ahead*

N. Wexler has been interested in following up on the results from the search for genetic modifiers in the Venezuela kindreds since they were published in 2008. As she explained, Housman suggested using whole genome sequencing to conduct a more refined search for modifiers and proposed waiting for the technology to become more affordable. In the meantime, N. Wexler applied for funding and obtained some support to begin sequencing samples of a father and two daughters who were amongst the 250 people found to have contributed to the linkage peak. Somatic cells from these individuals, with CAG repeat lengths between 40 and 70, have now been whole genome sequenced.

N. Wexler noted that the study could be continued and extended. Frozen brain tissues from many family members of these three people are available and could be examined. In addition, Hughes suggested performing whole genome sequencing on samples from the 250 individuals. Also, RNAi screens in animal models of HD could be used to test the effects of downregulating genes residing within the loci that yielded the highest LOD scores.

Participants also discussed an ongoing collaborative effort between the Gladstone Institute of Neurological Disease and the Institute for Systems Biology involving whole genome sequencing to find HD modifier genes. Jacqueline Jackson said she provided Steve Finkbeiner, the lead researcher, with approximately 100 samples from the Indiana repository. She noted that Finkbeiner requested extreme phenotypes and samples from multiple (at least three) generations of the same family. McDonald explained that including multi-generational samples helps minimize sequence errors. Participants were hopeful that this ambitious project will yield important new findings.

Searching for modifiers of HD in human iPSC cell lines is another promising avenue of research. Leading the HD iPSC Consortium, Thompson has already assembled a sizable collection of HD iPSC lines, with huntingtin alleles spanning a range of 17-180 CAG repeats. In addition, as noted at the HD2014 meeting, a European consortium is setting up to curate thousands of iPSC cell lines.

Participants also discussed potential non-genomic sources of HD modifiers, including epigenetic modifications and the microbiome. As suggested by Thompson, examining the Venezuela samples for epigenetic modifications could be valuable, particularly considering there is ample baseline information on human DNA methylation patterns. Yang agreed and added that his understanding is that epigenetic analyses are much more stable and reliable than transcriptome analyses. He further noted that, in a recent study, researchers were able to predict the age of an individual, within a three-year range, exclusively through epigenetic analyses.

Hughes acknowledged the power of such analyses but suggested that focusing on the genome, rather than the epigenome, should be the top priority given that the genome is less complicated and offers a more direct route towards developing therapies. In addition, Cha was concerned about the risk of obtaining a high number of false positives as a result of multiple-hypothesis testing.

Thompson and Yang stressed, however, that major advances have been made in epigenomic analyses, and statistical tools to deal with potential multiple-testing artifacts are available. Moreover, Thompson noted that epigenetic modifications are druggable. Shoulson added that, even if a particular modification isn't druggable, identifying it would still provide a valuable pointer to candidate modifier genes. Yang concluded that it would be valuable to examine the epigenomes of Venezuelan samples and correlate them with phenotypic profiles. Cha agreed and considered it worthwhile to investigate whether there are therapeutic options to modulate methylation patterns or chromosomal structure to ameliorate disease pathology.

Participants also mentioned the possibility of finding viral modifiers of HD, as noted by Weiner, or microbial modifiers, as noted by N. Wexler. Young pointed out that several studies have implicated the microbiome in neurological disorders, such as Parkinson's disease and Alzheimer's disease. Moreover, Paul Patterson and others have begun examining the role of the microbiome in HD.

As noted by N. Wexler, the biggest challenge moving forward with the search for HD modifiers, however, is money. Kurt Fischbeck suggested designing small-scale studies to have a better chance at getting funded. On the other hand, McDonald proposed looking for a visionary donor willing to fund a large project based on an understanding of the future implications of modifier studies, not only for HD, but for other diseases. A particularly compelling suggestion, put forth by Hughes, is to engage someone in the instrumentation industry. In particular, Hughes suggested contacting Jonathan Rothberg, a biotech entrepreneur in the field of massively parallel DNA sequencing. Rothberg is the founder of 454 Life Sciences Corp. and, more recently Ion Torrent, a company commercializing a new method of sequencing. Hughes noted Rothberg would probably be interested in funding a modifier search if his technology was used to do it.

### **Concluding thoughts**

Contributing to meaningful drug development emerged as a major goal for participants at the workshop. Chesselet and Bates underscored the importance of providing clinical researchers with solid data, including target validation, to justify the large investments required for conducting clinical trials. Fischbeck agreed and described a graphic that Maynard Olson once used to explain medical research: the path to treatments starts with the individuals afflicted by disease and eventually comes around full-circle to these individuals when a treatment is developed. In HD, Fischbeck considered, the research has covered a significant portion of the circle, and now the key to closing the final gap is to focus on the right drug and the right kind of clinical trial. As discussed at various points during the workshop, finding the right timing to administer different candidate treatments

will be key, as will be investigating the possibilities of treating HD pre-symptomatically. It is likely that many treatments will be much more effective if administered before major pathology has ensued.

Chase said he is in search of promising drug leads to support proof-of-concept trials. Moreover, Cha noted that the pharmaceutical industry will need to be engaged eventually and he hopes to motivate Merck to participate in this process. Fischbeck and Chase added that HD research might also benefit from supporting the development of drugs for symptomatic treatments, like tetrabenazine, particularly with the help of people like Kathleen Clarence-Smith and N. Wexler who worked very hard to facilitate the process of moving the drug through the pipeline and obtaining FDA approval. As noted by Fischbeck, performing clinical trials for these drugs is relatively easy and cheap as compared to running trials for treatments designed to cure the disease. And, as noted by Chase, palliative drugs can make a huge difference in the quality of life of individuals suffering from a disease, as illustrated by how L-dopa greatly helped people with Parkinson's disease when it came to market. Clarence-Smith agreed and said she is now interested in raising money for the testing and approval of drugs to enhance cognitive function in HD.

At the end of the workshop, participants shared their ideas of what they considered to be the most important next steps. Answers ranged from continuing the development and improvement of technologies for current candidate therapies in the pipeline, to accelerating the development of new therapies based on recently discovered targets, to deepening the understanding of HD and revealing new sources of therapeutic targets. As for the Hereditary Disease Foundation, it plans to continue to be a driver of HD research. Supporting and inspiring current and future scientists committed to finding a cure, it hopes to continue to live up to Gill Bates's enthusiastic statement, "The HDF has been with us all the way!"