Hereditary Disease Foundation

“Cure Hunters – Changing the Future”

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Scientists came together at the workshop “Cure Hunters – Changing the Future” to help move forward the Hereditary Disease Foundation’s vision of finding a cure for Huntington’s disease (HD). They reviewed the current state of HD research, considered new ways to deepen our cellular and molecular understanding of HD, and discussed potential new paths towards identifying and validating targets for therapeutic intervention.

The importance of improving clinical trial design and the tools available for tracking disease progression and drug efficacy in humans was highlighted. Participants also shared their ideas about advanced therapeutic candidates and what needs to be done to accelerate their development. Of particular interest was the discussion of a zinc finger protein designed by the biotech company Sangamo to shut down transcription of the mutant huntingtin gene. Moreover, an in-depth discussion of the issues regarding the delivery of silencers and repressors of huntingtin gene expression in human brains yielded new ideas to address the challenges of tissue specificity and spread, dosing, monitoring of target engagement, and safety.

Promising possibilities for identifying new targets and validating ones that have already been described emerged from discussions of huntingtin protein folding, aggregation, and clearance, as well as the concerted regulation of cellular stress responses. In addition, participants pointed out several downstream pathways as the source of promising target candidates, including the neuronal growth factor BDNF and its receptor TrkB, the nuclear hormone receptor PPAR-delta, and the transcription factor Nrf2.

Reviewing the basic biology of HD, participants stressed the importance of understanding the mis-splicing of mutant huntingtin and its implications in greater depth, as well as dissecting the dysfunctional neuronal circuitry associated with the disease. The importance of understanding the role of various brain regions beyond the striatum, including the cortex and thalamus, was stressed, as was the importance of striatal and cortical interneurons, cortical pyramidal neurons, and astrocytes. Participants also emphasized the need to understand the sequence of pathogenic alterations so that therapies can be delivered in a timely manner.

Advances in several technologies that promise to accelerate the understanding of HD and the search for a cure were also discussed. For example, participants described the generation of isogenic iPS cell lines expressing the huntingtin gene with a range of CAG repeats, a new method to analyze the transcriptome of single cells in combination with a robotic microscopy system, and the use of machine learning algorithms to analyze very large datasets derived from monitoring hundreds of cellular phenotypes in individual cells over time.

Changing the future of a family

The workshop offered participants the unique opportunity of learning about HD and its devastating consequences through the generous participation of an inspiring family in

which the father, Josh, has HD. The experiences shared by Josh and his wife, Rachel, informed participants and inspired them to keep searching for a cure.

Thirteen years ago, when Josh was 21 years old, he requested to be tested for HD at the army base where he was serving. His mother had been experiencing uncontrolled movements and other symptoms, and had been diagnosed recently with HD. Josh knew he had a chance of getting the disease. He also knew he could pass it on to his children and was particularly worried because Rachel was pregnant with their first child. Without any counseling, he was informed that he had an expanded repeat for the HD mutation. In a couple of days, he shared the news with Rachel and said he understood if she wanted to leave him.

Rachel didn't hesitate in responding that she would stick with him and together they would face the disease. Josh didn't come to terms with the diagnosis, however. It was a constant concern, always in the back of his mind, always tormenting him as he visited or talked with his mother or grandmother, both who suffer from HD. Yet Josh did nothing to prepare for the disease for five years. As Rachel explained, he was just not ready. "I never really adjusted to the news," said Josh.

Once Josh's symptoms surfaced in 2008, however, everything changed. Both Rachel and Josh were galvanized into action. They spent eight months doing paperwork and waiting for a response from the government to get disability payments for Josh. In the end, their claim was denied. So Rachel called and wrote to their political representatives, pushing hard to get the support they needed. She succeeded and now Josh is on disability pay and covered by Medicare.

Josh has painful tics in his neck and shoulders that often interfere with his sleep. He gets spasms in his back, sometimes so strong they knock him off his feet. He also suffers from unpredictable mood swings. The smallest thing, a spilled cup of coffee, can suddenly trigger a flare of anger or severe anxiety. The anxiety is the worst. It was the first symptom of HD he experienced five years ago and it has been with him ever since.

Josh had been a confident man, skilled at his work as a salesperson and manager in retail business. He was good at dealing with people and often gave speeches and trained new employees. But five years ago, when he was about to walk up to a podium to receive an award, he suddenly felt a surge of anxiety so intense it made him throw up and then pass out. The event marked the beginning of his and his family's grueling battle with HD.

Rachel, Josh, and their oldest daughter who is now 13, have become tireless and vocal advocates for individuals with HD and their families. They participate in support groups and have organized two fundraisers for the cause. Rachel even got a tattoo promoting the search for a cure for HD. She says it's a great way to start a conversation and educate people about the disease. At first, Josh said he was reluctant to go to HD meetings because it was so hard for him to see people in the later stages of the disease. But one time, over a couple of drinks, he had an inspiring conversation with a man at a more

advanced stage of HD who offered support and guidance. It deeply affected Josh and now he says he wants to do the same for others.

In addition, Josh is participating in several clinical studies, including the large ENROLL-HD study and some of its off-shoots. In a moving statement, he added that he would be willing to be a pioneer--or as Anne Young put it, “an astronaut”--for the clinical testing of new therapies. He said he would accept to undergo surgery and the delivery of candidate therapeutic agents to his brain. He clarified that he would do this, which would cause him great anxiety, not for his own sake, but for that of his three children.

Also inspiring was the description of how Rachel and Josh work together to cope with HD. Rachel spoke very highly of how Josh manages his mood swings and other everyday challenges, praising his remarkable understanding of his disease. In turn, Josh spoke about Rachel’s empathy, openness, and ability to step up and help with every aspect of their family life. Participants applauded the couple’s attitude and approach to living with HD. Yvette Bordelon, Josh’s neurologist who presented the family at the workshop, pointed out their insightful recognition of the disease and its challenges, their dedicated planning efforts, and their creative and constructive strategies. At the end of the workshop, Bordelon shared a message from Rachel thanking workshop participants for their efforts and noting that, for the first time, Josh is hopeful about the development of a cure. The opportunity he had of meeting and interacting with so many scientists who are working hard on HD made him feel encouraged and grateful.

Current clinical trials

Motivated by Josh’s and Rachel’s accounts, workshop participants reviewed the state of HD research and identified key issues that need to be addressed. Participants listed a number of therapeutic candidates currently in clinical trials, including Coenzyme Q10, creatine, deuterated tetrabenazine, the dopaminergic stabilizer ACR16, a vasopressin inhibitor (to reduce irritability), dimethyl fumarate which activates the nuclear-like factor 2 (Nrf2) pathway, phosphodiesterase-10 inhibitors, memantine which blocks the NMDA receptor, and the copper/zinc ionophore PBT-2.

Challenges
Unfortunately, the preclinical data supporting the use of several of these compounds are weak and there’s a scarcity of reliable outcome measures to track the compounds’ effectiveness. As noted by Jang-Ho Cha, in many instances, researchers are expecting a “home run,” an unequivocal positive result that will reveal the efficacy of a drug on the first attempt, despite the suboptimal conditions of the tests.

As noted by Anne Young, the variability associated with current clinical outcome measures (including the Unified Huntington’s Disease Rating Scale, in addition to a few motor tests used in Europe) is such that large numbers of participants are needed for studies to have enough power to provide clear results. Also, Young pointed out that most studies are comprised of participants with a wide range of symptoms, including some in

the very late stages of disease. This can obscure positive results from a responsive subpopulation.

Leslie Weiner pointed out that another difficulty is that the FDA requires clinical effectiveness and is resistant to accepting other measures for tracking disease, such as neuroimaging and behavioral tests, especially for phase III trials. Marie-Francoise Chesselet added that companies often try to follow the FDA's recommendations without proposing and pushing for changes, which results in the FDA becoming even more set in its ways.

Another problem is that companies seeking to save money and time often fail to follow recommendations put forth by scientific advisors. Gillian Bates said that the European HD Network (EHDN), of which she is a member, won't endorse studies which do not heed the EHDN's advice. Although a lack of endorsement is a powerful statement, it is not always sufficient to block the execution of poorly designed studies.

Solutions

As emphasized by Cha, it should be possible to get better clinical measures that reflect treatment effects reliably. A good clinical predictor would shrink costs substantially, reducing trial time and size. In-depth, solid clinical research is needed to achieve this goal.

As pointed out by Young, there are already several studies that promise to help move the field towards this objective. The Prospective Huntington's at Risk Observational Study (PHAROS) and PREDICT-HD 2.0, for example, are providing important information that is likely to lead to the development of better outcome measures. For example, a new insight derived from PHAROS, noted Young, is that behavioral changes in HD progress at a completely different rate than motor and cognitive changes. Whereas motor and cognitive deficits seem to intensify gradually with disease stage, behavioral alterations (such as apathy, depression, aggression) seem to emerge very early, before the commonly recognized symptomatic stage, and not change much over the course of the disease. Bordelon added that PREDICT-HD 2.0 is helping refine cognitive measures, as well as other indicators, of the disease.

Moreover, Gill Bates noted that the EHDN is running the REGISTRY study, a multi-national observational project that aims to study the natural history of HD, and has approximately 10,000 enrolled participants. Several clinical working groups focusing on different phenotypes, such as motor and psychiatric, are making progress in identifying clinical features that may be of use for therapy development. Fyodor Urnov added that the TRACK-HD study, led by Sarah Tabrizi, is also providing useful information, including the identification of HD-associated alterations that are measurable many years before the symptomatic stage.

Which of these collected data will ultimately yield robust outcome measures and how they will be used for clinical trials depends on several factors. For example, as noted by Cha, whether a therapy is designed as a disease-modifier or to target symptoms is

important. Young added that the predicted effect size of a therapy, patient population and repeat length are also critical factors to consider. Weiner added that, based on what is known about the natural progression of HD, he expects it will be difficult to assess therapeutic efficacy for most candidate agents in trials that are less than 5 years long.

Cha noted that futility trials—trials that are designed to be stopped when the interim results suggest they are unlikely to achieve statistical significance—help save time and money, but designing trials more carefully from the beginning could result in even greater benefits. Clinical trials that include biomarkers for monitoring target engagement and designed to yield useful information whether the results are positive or negative are much needed. Chesselet suggested recruiting researchers with clinical trial experience working on other diseases to improve trial design. Young added that having skilled people to run the trials was also needed.

Another strategy that might prove useful was suggested by William Yang based on recent studies of tetrabenazine, an anti-choreic medication. Tetrabenazine was originally tested as a drug to treat an HD symptom but is now being studied for its disease-modifying potential. So Yang wondered if, for some candidate treatments, it might be possible to apply this same strategy—run symptomatic trials first and then follow up with more costly and lengthy disease-modifying studies. If the results from the symptomatic trials are positive, there might be a greater impetus to undertake the trials to assess disease-modifying potential.

Participants also discussed the need to work with the FDA to adopt better outcome measures. Cha pointed out that the agency can be flexible in certain situations. For example, it recently accepted new clinical scales to monitor prodromal Alzheimer's disease, which do not include the standard cognitive and functional tests. Also it recently accepted non-symptomatic indicators—levels of Aß and tau in cerebrospinal fluid and plasma—as measurements for patient selection. Cha added that, recognizing the severity of HD, the FDA allowed in silico modeling, instead of the more usual clinical study, as an indicator of tetrabenazine's drug-drug interactions. Although the long clinical history of tetrabenazine helped push the drug through FDA approval, the agency's acceptance of modeling data is encouraging.

Cha explained that it takes a lot of preparatory work to approach the FDA with a new outcome measure, but the procedures are clear and straightforward. In brief, a protocol with supportive data needs to be submitted showing that the new measure tracks disease progression and can be used to monitor a particular therapy's effects. The protocol should be submitted well before compound testing is planned.

Participants noted that the use of imaging markers to track HD is one of the types of protocols that should soon be proposed to the FDA. Although the agency has been resistant to accept such markers, noted Weiner, Cha considered that if strong supportive data are obtained, as for multiple sclerosis, this might change. Weiner suggested performing imaging studies in every clinical trial, in order to collect data that may later serve as evidence for using a particular imaging measurement as a biomarker.

Magnetic resonance imaging (MRI) and positron emission tomography (PET) studies have already yielded some promising results. For example, Bordelon's team has used [18F]FDDNP to monitor a variety of amyloid aggregates, including HD aggregates which can be detected in the basal ganglia in presymptomatic individuals. Although signals are somewhat noisy, they increase with disease progression. Ongoing longitudinal studies should help determine the potential value of this marker. In addition, Bordelon is studying other PET markers (for neuroinflammation, glucose transporters and monoamine oxidase in astrocytes) that may also prove useful as biomarkers. As explained in more detail later, efforts to image huntingtin mRNA in the brain are also ongoing. This approach is expected to be particularly useful for monitoring therapies designed to reduce mutant huntingtin expression. Bordelon added that spectroscopy measurements should also be considered.

Participants pointed out that imaging biomarkers may also be useful to select patients for different clinical trials. For example, Young pointed out that AD researchers recently used imaging in a clinical trial designed to test an anti-amyloid candidate therapy, selecting only individuals with detectable amyloid (16% of people affected by AD don't have detectable amyloid). As noted in the previous section, targeted patient selection could help decrease the cost of clinical trials and make them much more informative.

At a more basic level, David Housman emphasized the need to identify additional cell-type specific and stage-specific markers to track disease, both for target validation and for tracking the effects of candidate therapies. Although well-defined cell type markers already exist (e.g., cell-specific receptors, transporters, signaling molecules), Housman opined that a much larger collection of markers to make very fine-tuned distinctions between neuronal cell types is needed—the equivalent of bar codes. How close one could get to having a bar code-like system is subject to debate, noted Chesselet, but participants agreed that broadening the collection of biomarkers is a desirable goal.

Moreover, identifying individuals who are willing to be "astronauts" or pioneers for testing new therapeutic agents will be important as well. Young noted that some of the therapeutic agents in the HD pipeline, such as repressors/silencers of huntingtin expression, may involve invasive procedures for delivery and monitoring. It will be important to identify individuals willing to undergo these interventions who are also good candidates to yield informative results (e.g., individuals like Josh, who are relatively young and at early- to mid-stages of disease).

Turning off mutant huntingtin expression

Sangamo's zinc finger repressor

A number of candidate therapies close to, or already in a preclinical testing phase, stand to benefit from the clinical trial improvements discussed above. For example, Fyodor Urnov described the design of proteins that selectively and potently shut down the transcription of the mutant huntingtin gene. These efforts, led by Sangamo Biosciences,

are at an advanced pre-clinical stage and the expectation is to start clinical studies in 2015.

As described in previous workshop reports, blocking the production of mutant huntingtin is the most straightforward approach to tackle HD and one of the most advanced candidate treatments in the pipeline. Most of these efforts have focused on gene silencing technologies that result in the degradation of huntingtin mRNA. Sangamo's approach, however, targets huntingtin mRNA production upstream. As explained by Urnov, Sangamo has developed a technology that allows them to engineer zinc finger protein domains that can bind to essentially any target sequence of DNA (a zinc finger is a small protein domain with a characteristic structure stabilized by zinc ions that enables it to recognize and bind DNA). By fusing these domains to other functional domains, such as a transcriptional repressor or activator, it is possible to manipulate gene expression in a highly targeted manner.

Searching for a zinc finger sequence that would specifically recognize the mutant huntingtin gene, Sangamo scientists tested large arrays of zinc finger proteins for their ability to bind to long stretches of CAG repeats. Initially, they obtained proteins that recognized the CAG repeat, but bound equally well to mutant and wildtype huntingtin alleles. They also obtained proteins that preferentially bound to the mutant allele, but had insufficient affinity to significantly block transcription when fused to a Krüppel-associated box (KRAB) repressor domain.

Ultimately, however, their engineering efforts paid off. The team identified a zinc finger protein that binds to CAG repeats cooperatively such that only a long stretch of CAGs results in robust binding. Its threshold for functional binding is 36 CAG repeats, strikingly in line with the boundary length at which CAG repeats in huntingtin cause fully penetrant disease. The protein has such low affinity for repeat lengths within the normal range that it has essentially no effect on the expression of the wildtype allele in cultured cells. Its robust binding to mutant alleles, on the other hand, results in a 5-100 fold decrease in transcription as assessed by RT-PCR and Western blots. The effect can be seen over a 300-fold concentration range. Urnov added that the team has obtained positive results delivering the protein using lentivirus or adeno-associated virus (AAV) in neuronal cells in culture and HD mouse models. The next step will be to deliver the vectors stereotactically to targeted brain regions, such as the striatum.

Based on these very encouraging results, Urnov and colleagues are planning to start work to fulfill the toxicology requirements necessary to run clinical trials. Their Investigational New Drug (IND) application is due in 2015, so the team has a full year to advance the toxicology studies. The plan is to have an informal meeting with FDA officials for a "pre-pre-IND" review of the toxicology in 2014.

Urnov explained that the huntingtin gene is unique in having a stretch of CAGs close to its transcriptional initiation site where the repressor can act and, thus, off-target effects are expected to be absent or minimal. Also, there are very few genes in the human genome with long CAG repeats that would be predicted to bind the repressor. Indeed,

Urnov noted that a global chromosome immunoprecipitation (ChIP) sequence analysis yielded negative results

Consistent with these expectations, Urnov noted that no off-target effects have been seen in cultured neurons (although some were observed in fibroblasts). Studies in mice are ongoing. So far, the researchers have obtained encouraging results, observing improvements in clasping, restoration of some medium spiny neuron markers, and no signs of apparent toxicity. Sangamo plans to extend these early findings and run more in-depth safety assessments using a large animal model. The CAG content of the genome of the animal of choice will be important. Unfortunately, noted Urnov, the CAG contents of mouse and non-human primate genomes are significantly different from that of the human genome.

Two of the key toxicology factors that will need to be assessed are: 1) the effect of the repressor on wildtype huntingtin levels and 2) the immunogenicity of the payload and/or capsid (see *Delivery of huntingtin repressors: Safety Considerations*). Ben Deverman noted that, if immunogenicity turns out to be a problem, miRNAs can be used to reduce expression of the construct in antigen-presenting cells, such as microglia. As noted by several participants, so far there have been no toxicity effects reported from the use of other gene silencing agents delivered via viral vectors in non-human primates (for up to 6 months), so that the general strategy appears to be safe. However, as pointed out by Chesselet, these tests have been performed in healthy animals. In a diseased brain with inflammation, the safety profile may be different.

Participants also discussed the design of clinical trials in humans. As noted by Urnov, Sangamo has experience in the clinical arena, having done extensive clinical work with AIDS, and a phase I trial would be relatively simple, involving 10 individuals (as is typical for gene therapy trials), without the need for a placebo group. The selection of participants will probably involve the identification of individuals in the relatively early stages of disease with CAG repeat lengths above 37. Carl Johnson pointed out that individuals with repeat lengths between 27 and 35 show partial penetrance of the disease and are sometimes classified as having “intermediate HD.” Given the binding characteristics of the Sangamo repressor, these individuals would not be good candidates for treatment. Urnov said the current expectation is that the Sangamo repressor will cover approximately 70 percent of the HD population.

When asked about his concerns taking the zinc finger repressor to the clinic, Urnov pointed to the levels of repression and the potential immunogenicity of the payload and capsid. So far, his team has observed robust repression in cells and mice, but as is the case with all current strategies to knock down huntingtin, making it work in the large human brain poses a challenge, as do immune responses, which are often hard to predict.

Delivery of huntingtin repressors

In discussing these and other challenges associated with delivery, participants turned their attention to vectors, target tissues, dosing, safety, timing, and monitoring target engagement in the broader context of the various therapies to reduce huntingtin

expression currently under development. As noted by Neil Aronin, one of the major challenges is the human brain's large size. The successes in rodent models of HD may not translate to humans unless techniques to distribute the repressors over large volumes of tissue are developed and/or particular brain areas/cell types are identified as key targets. To address these challenges, Aronin noted that several sheep models of HD have been developed (including fragment and full-length huntingtin models). His team has approximately 60 sheep and is expecting to almost double this number soon as the birthing season arrives.

One approach for dealing with a large brain is to deliver repressors to a localized region in the brain known to be particularly vulnerable to HD, such as the striatum. Although this has the advantage of not requiring coverage of a very large tissue area, it may not yield optimal results. First, it is increasingly apparent that multiple brain regions, in particular the cortex and thalamus, are critically involved in the disease process (see *Circuitry alterations in HD*). In addition, as pointed out by Aronin, most current approaches to deliver silencers to the human striatum involve injections into the putamen which is relatively accessible, but not the caudate, which requires more challenging lateral surgery. Aronin noted that Ann Graybiel considered that this type of limited intervention would fail to treat many aspects of HD, including problems with memory and depression.

On the other side of the spectrum is the approach of delivering silencers intrathecally, or in an even more widespread manner, intravenously. The advantages of this strategy are reduced invasivity and the potential for reducing mutant huntingtin expression across the entire brain, and in the case of systemic injection, in peripheral tissues as well. As noted by Ben Deverman, mutant huntingtin is known to disrupt metabolic processes in a wide variety of cells, including several peripheral tissues.

As summarized by Aronin, Isis Pharmaceuticals appears to have obtained reasonably good delivery of antisense oligonucleotides (ASOs) into the cortex via intrathecal delivery. Their current plan, noted Urnov, is to re-administer the ASOs every six months. Researchers working on expression-based repressors (such as shRNA, miRNA, zinc finger repressors) are examining ways to distribute these agents widely using intrathecal or intravenous delivery of viral vectors. Aronin noted that AAV9 is one of the favored vectors for this application because it crosses the blood-brain barrier and infects a fairly large subset of neurons (30-50%).

However, other AAV subtypes are also under investigation Beverly Davidson who has been running tests with AAV1 and AAV2, for example, has obtained reasonable neuronal infection efficiencies with these subtypes. Moreover, Lisa Stanek at Genzyme has used AAV2 to deliver a miRNA silencer with encouraging results. Deverman noted that AAV2 is efficient but cannot be concentrated as much as AAV8 or AAV9 because it tends to clump. Aronin added that AAV2/rh8 appears to be effective but does not spread much and AAVrh10 spreads widely, but infects glial cells primarily. Studies with AAV6, which shows lower immunogenicity than other serotypes, are also underway.

Tissue specificity and spread

But many challenges remain. One problem is the difficulty of specifically reaching key target cells while minimizing undesirable effects in non-target cells. A couple of new approaches to address these issues were presented by Deverman who is working in Paul Patterson's lab. Deverman explained that his team's initial goal was to enhance the tissue-specificity of gene expression by designing constructs with cell type-specific promoters. However, the researchers had trouble achieving the levels of specificity they were aiming for. So instead of promoting the expression of the silencer in target cells, they tried dampening expression in non-target cells. To achieve this, they included miRNA recognition sequences flanking the encoded silencer. The sequences were selected to hybridize with tissue-specific miRNAs produced in non-target cells, resulting in the degradation of the encoded RNA within those cells. The researchers have now run tests with constructs containing miRNA recognition sequences specific to several non-target peripheral tissues, including liver, pancreas, and muscle, resulting in a significant increase in brain specificity.

Deverman and his colleagues are also tackling the problem of tissue specificity using directed evolution to produce capsid variants with selective binding properties. The team initially tried to use rational design techniques, but discovered that the directed evolution strategy was more effective. Using libraries of 10 to 100 million different AAV variants, the researchers perform several rounds of selection to enrich for variants that more efficiently enter specific cell types. So far, Deverman and colleagues have generated viruses that enter the CNS efficiently and more selectively infect astrocytes. They are now searching for variants that more efficiently target forebrain neurons and could search for cortical- and striatal-specific variants as well. In principle, the approach could allow a fine-tuned delivery of silencers tailored to correcting the known disruptions in brain circuitry caused by HD (see *Circuitry alterations in HD*). At the same time, the intravenous delivery method offers widespread accessibility to tissues throughout the body, so that the intervention could reach peripheral targets as well.

Aronin added that viruses that are efficient at spreading retrogradely could help target repressors to neuronal circuits known to be particularly affected by HD. For example, AAV6 was recently reported to disseminate exclusively by retrograde axonal transport and several groups, including Aronin's and Yang's, are interested in using if for HD research and therapeutics. Aronin cautioned that the original report describing AAV6's tropism relied on a small number of animals but, if the data are confirmed, a single striatal infusion of this vector may permit efficient transduction of cortical and thalamic neurons in significant tissue volumes that otherwise would be difficult to achieve. Deverman added that he has begun a collaboration to optimize retrograde transport of AAV using directed evolution.

As noted by Carl Johnson, however, using retrograde transport to target corticostriatal and thalamostriatal circuits fails to address the altered function of cortical and striatal interneurons which appear to play a key role in HD pathology (see *Circuitry alterations in HD*). Also, as noted by Chesselet, it will be important to determine whether these

various efforts to enhance delivery will work in HD brains as they work in healthy brains. HD pathogenesis, in particular alterations in axonal transport, may affect viral spread.

Dosing

Another issue discussed by participants is that the knock down efficiency achieved so far has been limited to approximately 50 percent and the fundamental reason for this is unknown. Several studies indicate that this reduction is sufficient to yield therapeutic benefits in animal models and, in fact, is desirable for non-allele specific approaches where it is important to preserve a minimum level of wildtype huntingtin expression, noted Chesselet. However, the extent and type of partial reduction needed for therapeutic purposes are unknown. As pointed out by Ai Yamamoto, it is unclear if only a fraction of cells are being functionally infected, or whether the knockdown in each cell is partial, or a combination of both. Also, as noted by Yang, it is unclear how much individual neurons are improving as a result of the treatment. It is possible that all cells improve similarly, or conversely, that only a few cells improve substantially and this is sufficient to provide symptomatic benefits. Indeed, Yang's team has data indicating that improvement is non-cell autonomous—reducing mutant huntingtin in some medium spiny neurons is beneficial to neighboring, non-treated neurons.

Resolving these open questions—defining how much huntingtin reduction is therapeutically needed and where, as well as how effective particular repressors and delivery systems are in individual cells and as a percentage of a cell population—will be important for optimizing clinical trials. If initial clinical studies yield negative results, noted Cha, the answers to these questions will be critical to determine whether or not it is worthwhile to run subsequent tests at higher doses, for example. In addition, solid dosing information will be valuable for assessing improvements in HD which is slow and progressive, as Cha said, like rust. The goal would be to maximize the dose of each candidate agent, while keeping it within the boundaries of safety, in order to have the best chance of detecting positive effects.

Participants noted that efforts to address these and other dosing questions are underway. For example, Leslie Thompson explained that her team and others are using induced pluripotent stem (iPS) cells to systematically assess the maximum amount that huntingtin levels can be lowered without causing toxicity. In addition, Deverman proposed a way to address the question of the knockdown ceiling effect using a Cre-lox system. Viruses loaded with the Cre recombinase could be administered to BAC-HD mice carrying a mutant huntingtin exon 1 construct flanked by loxP sites in the target tissue. The "floxed" exon 1 would serve as a relatively clean reporter of the effectiveness of viral infection since it would be completely knocked out in response to successful delivery of the viral cargo. Quantifying exon 1 reduction in the target tissue would then provide a direct measure of the effectiveness of the virus as a delivery system to that target, independent of other complicating factors.

Monitoring target engagement

In addition to obtaining this type of preclinical data, participants agreed that obtaining dosing information from biomarkers of target engagement in initial clinical studies will

be very important. Rainer Kuhn noted that tracking huntingtin protein levels in humans is difficult, but his team has made some progress developing assays that can detect the protein in plasma and cerebrospinal fluid (CSF). In a small pilot study, his team detected increasing levels of huntingtin in CSF with disease progression. The researchers also observed, in plasma, higher levels of the protein in individuals with HD compared to controls (but couldn't detect a difference between premanifest and manifest disease). The sensitivity of the technique, said Kuhn, is in the femtomolar range. Kuhn also clarified that the assay in its current form does not distinguish between full-length huntingtin species and fragments, nor between wildtype and mutant forms of huntingtin.

Kuhn said it is unlikely his technique is sensitive enough to detect, in CSF, a reduction in huntingtin levels mediated by the delivery of a repressor to a localized brain region, such as the striatum. Young wondered if local sampling with pipettes or cannulas left for several days in the brains of treated individuals could circumvent this problem and provide valuable information, not only of the magnitude of reduction, but of its kinetics.

As an example of a possible scenario, Young described delivering 300-600 microliters of a solution containing viral vectors carrying Sangamo's zinc finger construct into the striatum, followed by partial withdrawal to pressure-infuse the vector into the cortex. A second bolus could be administered to another cortical location by changing the angle of the needle going in through the same skull hole. As suggested by David Housman, the choice of the location of the hole and the angle of the needle could be informed by the individual's particular phenotype. (Richard Faull has reported that, whereas some individuals suffer primarily from motor symptoms, others have more mood alterations, and these phenotypic differences correlate with the location of brain pathology.)

The intervention would be tracked using MRI and possibly PET imaging, with gadolinium included in the injection to monitor the initial delivery process. Subsequently, at regular intervals after the surgery, samples could be withdrawn and analyzed for huntingtin protein levels using Kuhn's assay. Young added that the study could be conducted with a group of ten volunteers who are at a fairly early stage of disease (at which time the disease usually progresses in a linear fashion) and who have been monitored closely for several years.

Several considerations were discussed regarding the feasibility and desirability of such an intervention. Cha opined that implanting cannulas would be desirable only if the measurements obtained were selected for their ability to directly inform future actions and/or provided unequivocal dosing information. He also suggested that the collection of samples for analysis should be carefully designed to obtain the most informative data, rather than guided by opportunistic considerations.

Participants also discussed the expected duration of the implantation. Aronin cautioned it could take as long as a month before changes in expression are detectable. However, Deverman and Urnov pointed out that the time frame might be much shorter since the sampling will occur at the injection site. Urnov noted that the half-life of huntingtin mRNA is only four hours and his team sees expression changes after repressor

administration in cell culture within 24 hours. Young added that implanting a device in a human brain for fairly extended periods has been done safely in the past (e.g., ventricular catheters and deep brain stimulating electrodes).

Participants also discussed other options for sampling and detection. Diane Merry proposed measuring mRNA levels and Yang added that extracting a very small sample, in the order of five cells, might be feasible for this approach, given the sensitivity of current RNASeq techniques.

Participants agreed that regardless of the details, the procedure should be validated in non-human primates or sheep before attempting it in humans. The validation process is particularly important given that there are several unknown parameters. For example, Bob Hughes pointed out that the information obtained through a cannula will be strictly local yet, as discussed above, the extent of spreading is a key factor in determining the effectiveness of a candidate repressor. Also, as noted by Gill Bates, the proposed analyses of huntingtin levels will lack a baseline measurement—the level of huntingtin before injection. Moreover, Deverman noted that the injection process itself will likely cause some degree of inflammation and potential changes in huntingtin measurements that won't be easily accounted for.

An alternative to measuring huntingtin levels by extracting samples, is to use brain imaging techniques. As explained by Merry, Eric Wickstrom has developed a technique using peptide-nucleic acid (PNA) chimeras to label and track specific mRNAs in living animals using positron emission tomography (PET). PNAs are oligonucleotide analogues in which the sugar phosphate backbone of a nucleic acid is replaced by a synthetic peptide backbone which is resistant to enzymatic cleavage.

Using hybridization modeling algorithms, Wickstrom has designed 12-mer PNA probes as markers for several types of tumors and is now working with Merry to develop a marker for huntingtin mRNA. Since PNAs cannot go through cell membranes, the researchers initially conjugated the huntingtin PNA to cell-penetrating peptides. However, the probes accumulated in clumps in the cytoplasm. Now the team has attached the IGF-1 ligand to enable receptor-mediated intracellular delivery. So far, reported Merry, the results are encouraging and the team is ready to assess whether they can detect huntingtin knockdown in cultured cells.

Merry expects the signal will correlate with huntingtin mRNA concentrations, based on Wickstrom's observations using PNA tumor markers. However, she also noted that huntingtin mRNA is expressed at much lower levels than the mRNAs Wickstrom has tracked in tumors. Hughes asked whether the probe might interfere with translation, but Merry said the probes are eliminated by the cell quickly, within 48 to 72 hours.

Safety considerations

Participants also discussed potential safety issues associated with delivering gene expression repressors using viral vectors. As noted by several participants, so far there have been no toxicity effects reported from delivering gene silencers via viral vectors in

non-human primates (for up to 6 months), so that the general strategy appears to be safe, at least in animal models.

However, different vectors and payloads could elicit different responses, and human immune responses can be difficult to predict. One concern is that a damaging and/or neutralizing immune response may be elicited against particular viral capsids or payloads. Indeed, Aronin noted that his studies with sheep indicate that these animals have immuno-neutralizing antibodies for some AAV serotypes in their blood even before the therapeutic vectors are delivered. Leslie Weiner added that the brain is not an immunopriviliged tissue such that, even therapeutic approaches that involve direct delivery to the brain, may elicit undesirable immune responses. Moreover, Chesselet noted that in a diseased brain with inflammation, such as occurs in HD, a harmful immune response may be more readily triggered than in a healthy brain.

Deverman noted that the directed evolution approach that his team has been working on could help address these problems. In principle, the technique allows one to select for almost any desirable characteristic that can be monitored in a viral vector, including immunogenicity. Thus, viruses that evade common antibodies could be selected for. Also, Deverman noted that specific miRNA constructs could be added to the viral payload to help reduce expression in antigen-presenting cells, such as microglia.

Participants also briefly discussed the possibility of generating constructs that express huntingtin repressors in an inducible manner. The potential risks of introducing DNA sequences that are permanently switched on and cannot be regulated have long been recognized. Thus, some laboratories, such as Patterson's, are working towards creating inducible-expression systems. Deverman noted that, in particular, they are trying to regulate gene expression by including a tetracycline response element upstream of the promoter so that tetracycline/doxycycline can be used to control expression. Aronin cautioned that, although potentially useful, there is scant information on the effects of these compounds on the human brain.

Aronin described another issue of potential concern. His team recently discovered that, at least in sheep, the liver and kidneys actively take up AAV. The researchers injected sheep striata with AAV9 and AAV/rh8 and found that the viruses were being shed from many organs for up to a month after injection, most dramatically from wool via the sebaceous glands at the base of their hair follicles. This shedding occurred even though there was no apparent leakage of the vector into the bloodstream during the injection as assessed by a gadolinium tracer.

Aronin's team is now beginning to perform histological analyses of the brains of sheep treated with vectors carrying anti-huntingtin siRNAs. At one month post-injection, the histology looks good, said Aronin, but he is particularly interested in looking at the six month samples. Aronin also noted that he has seen some microglial response, but no cytokine elevation. Moving forward, his team wants to focus on parameters that the FDA will find useful to evaluate toxicity.

In conclusion, there are many challenges to delivering repressors/silencers of mutant huntingtin expression, but there are also many efforts underway to address them. And as noted by Aronin, if the shortcomings of individual strategies turn out to be too difficult to address, combining approaches to circumvent the limitations of one with the strengths of another might ultimately yield effective combination therapies.

Additional therapeutic candidates

Protein folding

Participants also discussed potential targets for therapeutic intervention downstream of huntingtin expression. For example, Steven Finkbeiner noted that his team has been examining the folding of aggregation-prone proteins as a target. In a screen of 100,000 compounds, the researchers identified 72 hits that "correct" protein structure. In silico analyses are now underway and the team has secured an early translational grant to collaborate with a biotech company in efforts to develop therapeutic candidates based on these findings.

The rational design of molecules that modify huntingtin folding might also be possible in the future as advances are made towards elucidating the protein's structure. For example, new insights into huntingtin's structure might soon emerge from experiments Gwen Owens in Pamela Bjorkman's lab has designed to make huntingtin crystals in space. Over the past two decades, several research teams have tried to elucidate huntingtin's structure, but have run into difficulties because of the protein's huge size and propensity to aggregate. Bjorkman's lab, for example, has been unable to obtain huntingtin crystals that are large enough to yield informative results by X-ray crystallography despite setting up more than a 100,000 different individual experiments.

But now the team hopes to get crystals of much higher quality by running crystallization experiments in microgravity conditions, on the International Space Station. As explained by Owens, experiments by others have shown that protein crystals grown in space are often much larger (10 to 20 times) and of higher quality than those produced on Earth. So Owens and colleagues are optimizing conditions and putting together a number of huntingtin samples to send to space on February 24th, including exon 1 fragments with polyglutamine stretches in the range of 16 to 72, exon 1 fragments bound to an anti-CAG antibody, the first 500 amino acids of huntingtin and a fragment that is approximately half of the full-length species. If the results are positive, they will be able to send more samples on a subsequent flight.

Aggregation

Understanding how other cellular proteins regulate huntingtin folding and aggregation could also lead to new therapeutic options. For example, Leslie Thompson described her studies in collaboration with Judith Frydman on *TRiC* (TCP-1 Ring *Complex*, also called *CCT* for chaperonin containing TCP-1). TRiC is a chaperonin that is essential for folding many proteins, especially those with complex topologies, high beta-sheet content and a propensity to aggregate. Frydman found that purified TRiC directly blocks huntingtin aggregation and even a small portion of a single subunit of the TRiC double-ring

complex can remodel huntingtin aggregates. After receiving a sample of the pure protein which she thought was coupled to a nanoparticle to enhance uptake into cells, Thompson discovered that TRiC is, in fact, a cell penetrating peptide, capable of entering the cytoplasm and nucleus of PC12 cells in culture on its own. In addition, the researchers found that TRiC produced by one cell can influence neighboring cells via secretion. Carl Johnson cautioned, however, that many of the cell-penetrating peptides identified to date work only in vitro. Thompson acknowledged this limitation and added that it is not yet known whether the peptide can penetrate the blood-brain barrier.

Nevertheless, TRiC is emerging as a promising therapeutic candidate. Thompson and colleagues found that, after injection into the striatum, the protein persists for 2-3 weeks and mediates a robust reduction in huntingtin aggregation. In addition, Thompson noted that behavioral experiments conducted by William Yang and Finkbeiner, and reproduced by others, have yielded very positive results. One potential therapeutic approach, noted Thompson, would be to transplant stem cells that express and secrete TRiC or, as suggested by Johnson, engineer endothelial cells to pump out the protein. Another option to regulate huntingtin folding via TRiC, noted Yang, is through the manipulation of vaccinia-related kinase 2 (VRK2). VRK2 is an enzyme that negatively regulates TRiC function through enhancement of its proteosomal degradation, as recently described by Kyong-Tai Kim and colleagues. Indeed, Kim observed that siRNA-mediated knockdown of VRK2 enhances TRiC protein stability and decreases polyQ aggregation.

Also worth considering are the kinetics of huntingtin aggregation. Thompson pointed out, for example, that mutant huntingtin aggregation can be accelerated by the addition of extracellular polyglutamine oligomers, R6/2 lysates, or postmortem cerebrospinal fluid samples. Similar observations have been made in models of other neurodegenerative disorders. The underlying mechanism is not yet understood, but one possibility is that the proteins are gaining access to the cytoplasm and acting as seeds or prion-like proteins. Alternatively, the stress caused by their extracellular presence might trigger increased intracellular aggregation. Thompson's team is currently examining the linearity of the process and how it can be modulated by TRiC.

Another strategy for targeting huntingtin aggregation was described by Gill Bates who recently published data showing that reduction of histone deacetylase (HDAC) 4 delays cytoplasmic aggregate formation in HD mouse models. As explained by Bates, her team became interested in HDAC4 after observing that suberoylanilide hydroxamic acid (SAHA) ameliorates motor impairment in R6/2 mice, reduces aggregate load in the cortex and brain stem, and restores mRNA levels of brain-derived neurotrophic factor (BDNF) in the cortex. SAHA has several effects on cells, but the researchers identified the reduction of HDAC4, at the protein and not the RNA levels, as the one that may underlie the observed changes in R6/2 mice.

The normal role of HDAC4 in neurons is unclear. Bates pointed out that, despite its name, it has been shown that HDAC4 and the other class 2a HDACs have deacetylase domains rendered inactive through a tyrosine to histidine substitution. However, it does act as a transcriptional repressor and in muscle is a repressor of MEF2. It also plays an

important role in bone physiology because HDAC4 knockout mice die of developmental bone defects. Its role in neurons, however, remains unknown. Regardless of these uncertainties, in HD, the protein seems to associate with huntingtin in a manner dependent on polyglutamine length, co-localize with cytoplasmic inclusions, and co-immunoprecipitate with mutant huntingtin. In addition, the researchers found that genetic reduction of HDAC4 delays cytoplasmic aggregate formation, restores BDNF mRNA levels, and rescues neuronal and cortico-striatal synaptic function in HD mouse models. This is accompanied by an improvement in motor coordination, neurological phenotypes, and increased lifespan.

HDAC4 has several glutamine-rich regions and can aggregate on its own. Thus, the data are consistent with a paradigm in which HDAC4 accelerates the aggregation of mutant huntingtin. Decreasing the levels of HDAC4 reduces huntingtin aggregation in the cytoplasm but not in the nucleus. This is consistent with the steady state levels of HDAC4 in the brain, which are cytoplasmic. Furthermore, its reduction has no effect on global transcriptional dysregulation (the only transcriptional change detected was in BDNF) and does not normalize the transcription profiles of HD mice. Therefore, it ameliorated phenotypes with cytoplasmic and not with nuclear aggregates. Bates added that it was clear that both nuclear and cytoplasmic mutant huntingtin contributed to disease. In previous experiments, her lab had shown that mice with mutant huntingtin targeted to the nucleus had some HD phenotypes but were long-lived, whereas the survival of mice with mutant huntingtin in both the cytoplasm and nucleus is greatly reduced.

It seems unlikely that the beneficial effects of reducing HDAC4 in HD mouse models are related to the protein's deacetylase domain. To date, administration of inhibitors that target the deacetylase activity of the class 2a HDACs (which includes HDAC4, 5 and 7) has had no effect on huntingtin aggregation, but new inhibitors will continue to be tested as they are developed. To understand HDAC4's effects in particular cell types, Urnov suggested repressing HDAC4 expression in a targeted manner. Bates considered this a good idea.

It remains unclear if other HDACs might play a role in HD pathogenesis via effects on transcription. As noted by Al LaSpada, studies by Elizabeth Thomas and colleagues claimed that the HDAC inhibitor HDACi 4b, which preferentially targets HDAC1 and 3, ameliorates many HD-associated transcriptional abnormalities. However, a thorough study by Michael Levine and colleagues was unable to reproduce these results. As noted by several participants, the effectiveness of HDACi 4b delivery is unreliable because the compound is unstable in solution and has been shown to be unsuitable for *in vivo* use.

Cellular stress responses

Understanding how cells respond to the presence of aggregates was another key topic discussed at the workshop. Using photo-switchable proteins (which change color irreversibly when stimulated by light) to do optical pulse-labeling of mutant huntingtin, Finkbeiner has found great diversity in cells' responses to huntingtin aggregates. Not only has he observed turnover differences between cell types, but also between individual

neurons of the same type. Moreover, these differences seem to correlate well with vulnerability.

Finkbeiner added that tracking the fates of individual cells, his team has found that approximately half of the toxicity they observe can be explained by misfolding and the other half by the cells' responses to misfolding. In some cell types, like astrocytes, the presence of misfolded proteins induces a strong heat shock response while in others, such as neurons, the responses are weak. Examining iPS cells, the researchers noted that non-differentiated cells are high responders and remain so when differentiated into astrocytes, but not into neurons.

Finkbeiner suspects that a cell's state is key to determining its ability to recognize the stress of aggregate accumulation and activate an appropriate response. Future experiments in which Finkbeiner plans to use nanopippettes to take RNA samples from individual cells over time should shed light on this question (see Looking Ahead: *New Technologies*).

Studies from Rick Morimoto's lab support the idea that a cell's state is key to determining its response to stressful events. Morimoto's team has found that the activation of all four stress regulatory pathways, including the heat shock response (HSR), depends on several factors and, in particular, the developmental stage of an organism. Recent data from his group revealed a dramatic shut down of all stress responses between 8 and 12 hours after *C. elegans* becomes an adult. Morimoto hypothesizes that this is because stress responses are optimized for the rapid differentiation of *C. elegans*. As predicted from these results, adult animals are indeed more susceptible to stress.

How aging and development affect stress responses in humans is unknown but, as suggested by Morimoto's studies, they are likely to be important. Work in mice by Bates and colleagues offers some clues about how changes in the HSR are associated with HD and how age factors into the equation. Bates had previously found a decreasing ability to activate the HSR with HD progression in both the R6/2 and Hdh Q150 mouse models. This was associated with a reduction in HSF1 binding and altered chromatin architecture. Extending this work, Bates's team has now monitored the HSR in six brain regions, muscle, heart and liver in mice that are 3 and 22 months of age. The heat shock response was induced in peripheral tissues by heat shock (via a thermal blanket excluding the head) and by administration of the HSP90 inhibitor HSP990. HSP990 treatment was also used to inhibit HSP90 activity in the brain. Interestingly, they found no clear differences between young and old mice—the induction of heat shock factor 1 (HSF1) was the same, as were levels of Hsp70, 25 and 40. However, the team did find tissue-specific differences of interest in that the heart had a unique response pattern.

Bates highlighted the importance of understanding in greater depth how disease stage affects stress responses. Her team, for example, observed that an inhibitor of HSP90 improved huntingtin aggregate load, motor performance, and other HD-related phenotypes in the R6/2 mouse model. However, these beneficial effects were transient

and diminished with disease progression, most likely because of the concurrent HD-associated impairment of the HSR.

The importance of how cells respond to an aggregation-prone protein like mutant huntingtin was also highlighted by studies showing large effects derived from differences in genetic backgrounds. For example, Morimoto noted that a Hawaiian strain of *C. elegans* is particularly resistant to toxicity caused by mutant huntingtin. His team is currently sequencing modifier alleles that might underlie this capability. Robert Hughes added that his team has observed differences between two yeast strains—a lab strain and a strain collected from the wild. The wild strain accumulates aggregates much more readily than the lab strain and the progeny show a ten-fold range in aggregation levels. Pinpointing the genetic differences that cause this variability may be challenging because the genomes differ greatly, by approximately one percent.

As noted by Ai Yamamoto, discerning whether there are different types of aggregates within individual cells could also help deepen understanding of cells' responses to aggregate accumulation. Morimoto agreed and noted that his lab has been conducting screens for enhancers and suppressors of huntingtin aggregation and it is clear that suppressing aggregation does not always reduce toxicity (only about a third of the suppressors reduced toxicity, another third increased toxicity and the other third were neutral). Indeed, his team finds that there is no absolute correlation between aggregation, lifespan and toxicity. Finkbeiner noted that a new microscope system with nanometer resolution may enable his team to resolve different types of aggregates based on their morphology and/or location. Coupling these observations with machine learning algorithms his team is developing in collaboration with Google may reveal important functional correlations (see Looking Ahead: *New Technologies*).

Participants also discussed the potential mechanisms underlying the regulation of stress responses. Morimoto noted that the stress response shut-off in *C. elegans* adults appears to be mediated by an epigenetic, chromatin-based mechanism. In addition, Bates pointed out that the only parameter her team has found to correlate with the disruption of the HSR in HD is a change in chromatin acetylation patterns. As Cha noted, these data support studies investigating the inhibition of the subclasses of HDACs that deacetylate histones as potential therapeutic candidates.

Another aspect of the mechanism underlying stress response regulation is the recent discovery that cell-cell communication is involved in activating these responses. Morimoto noted that his team has found that if a misfolded protein is expressed in one tissue, paracrine or endocrine factors are secreted that trigger transcriptional changes that activate stress responses in other tissues. Several years ago, Morimoto had observed hints of this non-cell autonomous phenomenon when cells were ablated in non-neural tissues and has now identified instances of neuronal cell-cell communication, including trans-neuronal chaperone signaling. To extend these findings, the team is now examining which neuron types are most important for sending these signals.

In conclusion, many factors come together to determine what the effects of an aggregation-prone protein like mutant huntingtin will be. Age, disease stage, epigenetic factors, cell-cell signaling, genetic modifiers, environmental factors and the length of huntingtin's polyglutamine stretch all influence the outcome. In many cases, cells are able to maintain proteostasis in response to acute damage, but are eventually overwhelmed if the insult is chronic. Modifier screens, such as those being conducted by Morimoto and Hughes, should help shed light on the key factors involved and how they interact. Perhaps not surprisingly, Morimoto noted that the vast majority of the modifiers his team has identified so far are genes involved in proteostasis.

Clearance

One of the main functions regulated by stress response systems is protein clearance, including autophagy and proteasome activation. Increasing attention is being directed to these processes as potential therapeutic targets. For example, Finkbeiner reported that his team is partnering with Nanosyn to develop small molecules that induce autophagy specifically in neurons. This work is important in the context of HD because autophagy has been shown to be a key mechanism for clearing aggregated proteins, including mutant huntingtin. Finkbeiner explained that his team used an in silico approach to develop a model pharmacophore of neuronal autophagy inducers and then rationally design a collection of structural analogs predicted to be strong, specific inducers. Working with medicinal chemists to reduce neuroleptic secondary effects, the researchers have now identified potent inducers of autophagy (5 nM) that do not induce these effects. Preliminary tests in a model of amyotrophic lateral sclerosis yielded encouraging results.

Enhancing the activation of proteasomes and the delivery of huntingtin fragments to proteasomes are also being pursued as potential therapeutic targets. As explained by Finkbeiner, eliminating huntingtin via proteasomes, before it aggregates, could help protect cells from aggregate accumulation and toxicity. As suggested by findings from Ron Kopito's lab, Finkbeiner suspects that aggregated huntingtin fragments do not clog proteasomes, but rather HD causes alterations in the kinetics of the cellular proteostatic network such that activating proteasomes could prove beneficial. Following up on this idea, Finkbeiner's team is generating small molecules that activate proteasomes and identifying sequences that can target mutant huntingtin to proteasomes. In support of the value of targeting proteasome clearance, Finkbeiner noted that early data emerging from his ongoing search for HD modifiers identified a proteasome gene variant (see Looking Ahead: *Modifier Searches*).

Thompson added that targeting huntingtin's post-translational modifications is another approach to enhancing proteasome-mediated clearance. For example, huntingtin SUMOylation, a post-translational modification regulated by serine phosphorylation, affects huntingtin clearance and can be modulated to enhance neuroprotection. In particular, 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. 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. Thus, PIAS1, serine phosphorylation and SUMOylation are emerging as potential drug targets for HD. And because these targets are also emerging as relevant in cancer, HD may benefit from advances in the cancer field.

Downstream targets

Participants also briefly discussed the therapeutic potential of targets that are downstream of huntingtin. For example, HD is characterized by a reduction in cortical brain-derived neurotrophic factor (BDNF) production and transport to the striatum, so efforts to enhance the signaling of the BNDF receptor TrkB are underway. In particular, Al LaSpada described a recent article by Frank Longo and colleagues indicating that LM22A-4, a small molecule ligand reported to specifically enhance TrkB signaling, reduced several HD pathologies in the striatum of R6/2 and BACHD mice. The reported effects of LM22A-4 included the amelioration of decreased DARPP-32 levels, neurite degeneration of parvalbumin-containing interneurons, inflammation, and intranuclear huntingtin aggregates. The BDNF mimetic was also reported to reduce cortical aggregates and prevent deficits in the dendritic spine density of medium spiny neurons.

Participants agreed that the reported results were impressive, but Rainer Kuhn and Finkbeiner pointed out that they have not been reproduced by other teams. Indeed, a recent paper by CHDI researchers reported that LM22A-4 does not protect striatal neurons from cell death induced by mutant huntingtin, and that it does not activate the TrkB receptor in vivo. Finkbeiner added that LM22A-4 has very poor brain penetration. Participants emphasized the importance of replicating results by independent groups.

Participants also briefly discussed the therapeutic potential of modulating peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptor proteins that function as transcription factors. As explained by LaSpada, drugs that modify the activities of these proteins are being tested for their potential benefits in several neurodegenerative diseases. Most efforts to date have focused on drugs that regulate PPAR-γ. For example, the PPAR-γ agonist rosiglitazone was recently described to have neuroprotective effects in a knockin mouse model of HD and another PPAR agonist thought to act through PPAR-γ, bexarotene, has been reported to ameliorate symptoms and pathology in Alzheimer's disease mouse models. Based on these findings, a phase II clinical trial is underway to test bexarotene in humans, noted Merry. However, as pointed out by LaSpada, the study in mice is controversial due to the lack of reproducibility of some of its findings.

Based on extensive work from his lab, LaSpada noted that PPAR-δ is likely a better target to pursue for HD therapies. LaSpada's team found that the activity of the peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), a regulator of mitochondrial biogenesis and oxidative stress, is decreased in HD. PGC-1α regulates lipid and glucose metabolism by activating PPAR-δ which is abundantly expressed in the brain. Moreover, PPAR-δ interacts with huntingtin protein and LaSpada found that a dominant-negative mutation of PPAR-δ results in an HD-like phenotype. His team is currently pursuing PPAR-δ agonists as potential therapeutic agents for HD. Carl Johnson

wondered if such a therapy combined with an intervention to reduce mutant huntingtin expression might be particularly effective.

Moreover, the modulation of another transcription factor, Nrf2, was discussed as an additional promising therapeutic target. As noted by Finkbeiner, Nrf2 regulates many cellular processes, including stress responses, the activities of proteasomes, autophagy, and chaperones, as well as inflammatory responses. It acts on immune cells, neurons and astrocytes, where it is particularly abundant. In addition, Finkbeiner noted that Nrf2 is the most potent modifier of mutant huntingtin toxicity his team has identified to date.

Leslie Thompson explained that Aleksey Kazantsev recently identified a compound that modulates Nrf2 and appears to be neuroprotective in HD and Parkinson's disease. The mechanistic details of how the compound contributes to neuroprotection have yet to be elucidated, but a pilot study indicates that the agent downregulates the inflammatory cytokine TNF-α in R6/2 mice. It is known that the compound penetrates the blood-brain barrier, and more comprehensive pharmacokinetic studies are underway.

The compound is particularly promising given that it is approximately ten times more potent and more selective than dimethyl fumarate (Tecfidera), a drug recently approved by the FDA to treat multiple sclerosis and which has been shown, as noted by Johnson, to ameliorate the HD phenotype in animal models. Trying to improve the novel compound's features even further, Finkbeiner's team is working on generating variants using structure-based drug design. Given Nrf2's wide-ranging, stress-associated functions, noted Finkbeiner, it will be a challenge to generate a compound that activates Nrf2 pathways without overly stressing cells, especially when using it in a chronic manner. Thompson added that dosing will probably have to be intermittent.

The basic biology of HD

Mis-spliced mutant huntingtin mRNA

Participants also discussed new findings regarding the basic biology of HD which have implications for therapy development. Bates described her work with David Housman showing that mutant huntingtin mRNA is spliced in mice (all tissues) and humans (cultured fibroblasts and postmortem brain tissue tested so far) 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 pathogenesis, considering its well-known toxicity. The finding 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.

Bates is now planning to generate mice that make full-length mutant huntingtin, but are not exposed to the mis-spliced exon 1 fragments, to assess the fragment's relative contribution to HD pathogenesis. 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.

As discussed in the October workshop, a transgenic mouse recently created by Scott Zeitlin offers 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 pathogenesis. 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, these mice had very few inclusions, even at 18 months of age. 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.

More recent data from Zeitlin's lab indicate that, in these mice, a new 5' splice site within the Lac operator is activated to produce a longer mRNA that includes a 5' fragment of intron 1 which contains stop codons. However, the researchers do not observe a protein fragment consistent with the use of these stop codons. They have also observed the production of a species with a deletion of the proline-rich-region at the 5' end of exon 1.

Zeitlin noted that he plans to investigate whether the exon 1 fragment plays a role in normal cell function. Although fragment production is low in wildtype cells, a small amount is detectable and Zeitlin wondered if it could play a role in development as does the spinocerebellar ataxia type 6 (SCA6) polypeptide which contains a poly-glutamine stretch and is derived from a bicistronic mRNA. Interestingly, Leslie Thompson noted that Clive Svendsen's group performed RNASeq analyses on ten different iPS cell lines with a range of CAG repeats and found that 170 of the 500 genes whose expression levels varied with CAG length were developmental markers.

Bates noted her team is interested in identifying the splicing factors involved in generating the exon 1 fragment. She 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. SRFS6 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.

Circuitry alterations in HD

Participants also discussed the importance of understanding the wide-ranging effects of HD toxicity and, in particular, how HD alters neuronal circuits. For many years, HD

research focused on the striatal medium spiny neurons because, as explained by Michael Levine, these cells' pathology is more evident and the cells are easier to identify than other vulnerable cells. However, many new lines of evidence indicate that other brain areas, including the cortex and thalamus, and other cell types, including interneurons, cortical pyramidal neurons, and astrocytes, play key roles in HD pathogenesis.

Summarizing some of the recent findings, Levine noted that data from his lab and George Rebec's indicate the cortex suffers from functional alterations at an early stage. Levine discovered that cortico-striatal pathways are disconnected in the disease process and this disconnection begins early. He also noted that a change in the excitation balance in the cortex occurs first and is followed by alterations in striatal electrophysiological responses. Consistent with these findings, Yang's team has found that HD-mediated disruption of cortical function prevents the increase in inhibitory postsynaptic currents in the striatum. He also found that many HD phenotypes are dependent on the expression of mutant huntingtin in the cortex. For example, his team abolished HD-associated anxiety in BACHD mice by switching off mutant huntingtin only in the cortex. Even to alleviate motor symptoms, classically associated with striatal function, Yang found he had to switch off both striatal and cortical expression of the mutant protein.

Jang-Ho Cha wondered if analyzing EEG recordings, perhaps using Fourier analysis, might enable the early detection of these cortical abnormalities and serve as a biomarker of disease progression. Yang pointed out that he has observed progressive increases in gamma frequency activity in one of his HD mouse models and similar results have been reported in other animal models of HD. Yang also noted that cortical activity is a good readout because it is relatively simple and analyzing EEG recordings from specific cortical areas could optimize the sensitivity of this approach. Cha added that implanting electrodes might be desirable if the information obtained could help guide treatments.

As noted by Marina Chicurel, the thalamus has also been implicated as a key brain area affected by HD at an early stage. Studies from Anton Reiner's lab, for example, indicate that thalamostriatal axospinous terminals in HD knockin mice are reduced even before defects in corticostriatal pathways are evident. The alteration may reflect a development defect and may help explain early motor defects in premanifest HD. Levine noted that his group is using optogenetics to examine thalamic pathways which activate striatal NMDA receptors more effectively than cortical pathways.

Participants also emphasized the importance of striatal and cortical interneurons in HD pathogenesis. Levine explained that even small populations of interneurons can have large effects on the excitation balance of the cortex and striatum. His team has observed changes in the physiology of both parvalbuminergic and somatostatinergic interneurons with the activation of the former resulting in enhanced inhibitory responses in HD and, conversely, the activation of the latter resulting in a small decrease in inhibitory responses.

Earlier studies had focused on somatostatin neurons because the somatostatin marker was the only one available to identify striatal interneurons. But more recent studies indicate

parvalbuminergic neurons are also importantly involved in the disease process. For example, Levine noted that Anton Reiner and Richard Faull recently reported that a large decrease in striatal parvalbuminergic interneurons occurs in HD with advancing disease grade and this reduction is likely an important contributor to HD dystonia.

Levine suspects that a change in the cortical excitation-inhibition balance occurs first, which then fuels striatal alterations. Chesselet added that the fast-firing parvalbuminergic neurons mature before the medium spiny neurons, so the former might be affected by mutant huntingtin first and, in turn, affect the maturation of the latter. Furthermore, Levine noted that the observed increase in extrasynaptic NMDA receptors in medium spiny neurons is likely to accentuate this disruption. Participants agreed that investigating the roles of both striatal and cortical interneurons in greater depth is a top priority and Levine encouraged the generation of interneurons derived from iPS cells.

The cell and molecular biology of how synapses and other cell-cell interactions are altered in HD was also discussed. Levine noted that the mechanism by which the cortico-striatal pathway becomes disconnected in HD is unknown, but he hypothesized that striatal dendritic spines might change first since spines are dynamic structures and studies have shown a decrease in dendritic spines and a thinning of dendrite branches caused by HD in striatal medium spiny neurons. If the axon terminals remain in their original locations, they might then activate extrasynaptic NMDA receptors, which have been reported by Lynn Raymond to have increased levels of signaling and expression in HD. As noted by Aronin, however, other anatomical studies have shown a proliferation of striatal dendritic spines and dendrite elongation associated with HD. As discussed below, this apparent inconsistency might be explained by differences in disease stage.

As noted by Yang, recent data from his lab suggest that presynaptic terminals in both the cortex and striatum of HD brains might be engulfed by microglia. Yang began investigating the fate of these axon terminals after learning of observations from Ben Barres's lab indicating that microglia phagocytose presynaptic inputs as a mechanism of pruning during development. The process is dependent upon the microglia-specific phagocytic signaling pathway and involves complement receptor 3(CR3)/C3. Yang discovered that striatal and cortical terminals in two mouse models of HD are tagged for complement-mediated removal in a manner consistent with microglial engulfment. To extend these observations, Yang is now conducting genetic tests.

Clarifying how synapses are disconnected and understanding the timing of these cellular events will be important for developing therapies. The options for glutamate-based therapeutic interventions, for example, will be very different depending on the type and timing of these alterations, including whether they involve the retraction/destruction of axonal terminals, dendritic spines, or both.

In addition, the role of other cells, such as astrocytes will be important to ascertain. As noted by several participants, a few studies have implicated astrocyte alterations in the disruption of synaptic function. For example, Johnson noted that Michelle Gray recently reported augmented glutamate release through Ca(2+)-dependent exocytosis from

BACHD astrocytes. Yang added that astrocytes may also affect neuronal function in HD through their roles in providing neurotrophic support, inflammation, and neuronal metabolism, among others. Hughes agreed and noted that a substantial amount of the ATP generated in the brain derives from the lactate astrocytes deliver to neurons. Yang suggested removing mutant huntingtin specifically from astrocytes using a Cre/lox system to help dissect the contribution of these cells to pathology. He also noted that some scientists are working on addressing this question by transplanting human HD astrocytes into mouse brains.

Non-specific cell-cell interactions might also be important in defining the progression of HD pathology. Cells that are physically close to damaged cells might become dysfunctional. As noted by Aronin, this type of self-sustaining damage is well-documented in the liver where a localized insult can lead to more widespread disease. Yang noted that, indeed, his group has observed increased pyramidal cell loss near sites of interneuron loss. And as previously mentioned, recent data suggest that extracellular misfolded huntingtin may act as a prion protein, inducing enhanced aggregation in exposed cells (see "Additional therapeutic candidates: *Aggregation"***)**. Furthermore, Morimoto and his team have found that paracrine and endocrine factors are secreted by cells harboring misfolded proteins that activate stress responses in other cells (see "Additional therapeutic candidates: *Cellular stress responses*"). Thus, as suggested by Aronin, it is likely that early interventions that contain the damage caused by HD will be most effective.

Participants agreed and added that determining the optimal timing for some treatments might be more complicated. For example, treatments that modulate glutamate or dopamine could have less beneficial, or even harmful, effects if not timed carefully. Not only do cell and synaptic loss come into play, but changes in cell physiology are also important. Levine noted that in BAC and YAC mouse models of HD, glutamate-mediated excitation in the cortico-striatal pathway is increased early in the disease and diminishes later. Thus, glutamate blockers are expected to be beneficial in the pre-symptomatic stages of disease, but potentially harmful later on. Levine also noted that studies from George Rebec's group indicate a biphasic change in dopamine transmission as well—at early stages of disease, dopamine levels are elevated but become depressed later on. Housman added that, in humans, D2 dopamine receptor levels decrease before symptoms ensue. This decrease, noted Levine, is expected to result in increased glutamate release. Yet another complicating factor is that D2 receptors are found at various pre- and post-synaptic sites in the striatum and cortex, mediating different functions. Anne Young noted that, from a clinical perspective, medications that regulate dopamine transmission, such as tetrabenazine which blocks dopamine from getting into neurotransmitter vesicles, tend to become less effective with HD progression. In conclusion, as noted by Chesselet, there is still much work to be done to understand the HD time course in humans and tailor treatments accordingly.

Looking Ahead

iPS Cells

Several new approaches may help address these questions and accelerate HD research and the development of treatments. As discussed in previous workshops, iPS cells have emerged as particularly powerful tools to examine HD mechanisms of pathogenesis, as well as test therapeutic candidates, in human cells. Finkbeiner and Thompson reported there are now between 70 and 100 iPS cell lines in the Coriell cell repository with approximately 30 derived from individuals with HD. Although a few scientists still question iPS cells' biological identity, most accept them as being equivalent to embryonic cells.

Using iPS cells experimentally, however, is not an easy task. As noted by Thompson, the differentiation factors are expensive, the differentiation protocols are suboptimal, the cells must be grown without antibiotics, chromosome stability must be monitored (although genomic instability is not worse than in other cell lines), there are differentiation stages that are very unstable, and it is very difficult to standardize the methodology. Moreover, as Finkbeiner jokingly said, "these cells want to differentiate, but not into what you want." Not surprisingly, as pointed out by Fyodor Urnov, the quality of iPS cells and their differentiated progeny is becoming an important biomedical issue as plans for using these cells for research and therapy continue to expand. Indeed, one company is working on developing good manufacturing process guidelines for this purpose.

The good news is that the protocols to maintain and differentiate iPS cells are rapidly improving. For example, Thompson noted that viral-based methods to re-program cells have now been replaced with episomal-based techniques which yield more consistent results. Episomes jump-start the re-programming process and are lost relatively quickly, with endogenous factors taking over. Also, as noted by Finkbeiner, there's increasing evidence that it might be possible to streamline the differentiation process by skipping some developmental stages in the dish. The original goal had been to re-create the differentiation of neurons based on what is known about the development of the nervous system, but now it appears one might be able to take short-cuts. In addition, the list of phenotypes to assess maturity is growing fast. Based on these developments, Finkbeiner suspects that within five years there will be differentiation protocols that are much simpler and faster.

To help this process of protocol optimization, Yang suggested examining how various cells differentiate in culture. For example, it is known that brain-derived progenitor cells follow a differentiation path to become neurons that is relatively preserved as compared to SH-SY5Y cells. Looking at the differences between these paths might reveal clues as to which stages are critical and which are not, as well as help define a "model" pathway to compare different iPS cell differentiation protocols. As Ai Yamamoto pointed out, we don't know what "real" neurons differentiating in a dish would look like, but these comparisons might help shed light on which steps are important/desirable.

Participants also discussed many of the powerful ways in which iPS cells are contributing, and may contribute in the future, to HD research. So far, researchers have used iPS-derived striatal neurons and forebrain-like neurons as cell models of HD. Finkbeiner explained that his team has used forebrain-like neurons—glutamatergic, excitatory neurons—for their screening experiments because these cells can be generated quickly and efficiently. Thompson added there is a long list of HD cell types that her team wants to generate, including astrocytes, endothelial cells (for studies of the blood-brain barrier) and other somatic cells. Furthermore, based on circuitry data which strongly implicate interneurons as key players in HD pathogenesis (see "The Basic Biology of HD: *Circuitry alterations in HD"*), Levine urged Thompson and Finkbeiner to generate these cells as well.

In some cases, there may be no need to go through the challenging process of creating differentiated cells, if the pathway being studied is present in progenitor cells, noted LaSpada. However, in many cases, as noted by Finkbeiner, it is still unclear which cell processes are relevant to HD pathogenesis. It is clear that HD phenotypes are absent from iPS cells, but the level of differentiation required to see key aspects of HD toxicity remains unknown.

A particularly exciting application of iPS cell technology is the recent generation of isogenic cell lines expressing huntingtin with different CAG repeat lengths, as described by Finkbeiner. With the help of Sangamo, researchers of the HD iPS consortium, used a zinc finger protein to edit the CAG expansion in individual cell lines. Finkbeiner noted that the editing was challenging, but the researchers have now succeeded in generating collections of lines with the same genetic background that vary only in their huntingtin CAG repeat lengths. Thus, the phenotypic effects of the repeat length can be studied independently, in isolation from the effects of individual genetic heterogeneity.

As described by Leslie Thompson, Clive Svendsen is collaborating with researchers from several labs to characterize ten of these newly generated isogenic lines, performing ChIP sequencing, RNA Seq, proteomics, and epigenetic analyses. Preliminary findings indicate that the expression of approximately 500 genes changes in a CAG-dependent manner and a large fraction (approximately 170) of these genes are involved in developmental processes.

Participants also discussed the possibility of examining other potential modifiers of HD toxicity using iPS cells. For example, Anne Young proposed studying how the age of the iPS cell donor may affect CAG repeat susceptibility. Finkbeiner pointed out, however, that re-programming donor cells to become iPS cells involves erasing all epigenetic modifications associated with aging. One possibility to circumvent this problem, he added, would be to re-program cells to an intermediate stage, in hopes of maintaining age-specific epigenetic profiles. Also, as pointed out by Hughes, examining mitochondria, whose genomes are not altered by the re-programming process, could yield interesting information. Paul Taylor added that investigating how cell passage number affects HD phenotypes could be of interest and Thompson noted there are protocols to "age" iPS cells in a dish.

Another suggestion was to examine a series of CAG-edited cell lines derived from people with different original CAG repeats (e.g., juvenile vs. adult). The studies Svendsen and colleagues are conducting with the CAG-edited lines described above, involve cells derived exclusively from individuals with juvenile HD. Participants also proposed examining iPS cells derived from people with similar CAG repeat lengths, but different ages of onset. It has been well established that HD age of onset is determined only partially by CAG repeat length and several studies to search for genetic modifiers of onset have been undertaken (see below).

Going forward, it will be important to run experiments with multiple iPS cell lines and multiple clones from each line. As noted by Johnson, many of the iPS cell studies performed to date have relied on the same, very small set of cell lines, including a control with a very low number of CAG repeats. Thompson explained that these few cell lines were the first to be widely available, but now that many more lines are being generated, it will be increasingly easier to include other lines.

Participants discussed whether, in the future, banking iPS cells might become a routine procedure. As noted by Al LaSpada, this may not prove necessary since drawing blood from a patient to obtain cells when needed is very simple. However, as noted by Thompson, a bank could provide a useful resource for obtaining parental and other familial information for research or medical purposes. As noted by Finkbeiner, the value of a bank will also depend on the future development of regenerative medicine.

A particularly powerful application of iPS cell technology was described by Finkbeiner who is using iPS-derived striatal cells to identify genes that modify HD age-of-onset in humans. The idea is to use the cell lines to screen for candidate genes identified through whole genome sequencing of DNA samples from HD families. The strength of the approach lies in: 1) the use of high-quality DNA samples derived from multiple generations of the same families, 2) the availability of detailed clinical records, and 3) the ability of Finkbeiner's team to monitor nearly 1000 cellular phenotypes in iPS cells. The strategy allows for the identification of genes with both small and large effects, including private modifiers that might not show up in classic genome-wide association studies.

Finkbeiner emphasized the value of the samples provided by the *National Research Roster for Huntington Disease Patients and Families* at Indiana University, a project supported by the Hereditary Disease Foundation. The project manages a DNA bank with approximately 4,000 samples from HD families in the U.S., including samples which helped map the HD gene. Nancy Wexler noted that they now also have registries for other neurodegenerative disorders, including Down's syndrome and Parkinson's disease. Importantly, noted Wexler, the rosters are set up so that samples can be banked for future use without undergoing genetic testing. This allows individuals who do not want to know their genetic status to donate samples.

In response to participants' questions, Finkbeiner explained that the samples' whole genomes (rather than exons only) are being sequenced because the funds for the project were earmarked for whole genome sequencing. This turns out to be advantageous,

however, because most candidate modifiers appear to be in non-coding regions. Indeed, Urnov pointed out that 90% of the hits derived from genome-wide associations studies are in regulatory regions.

Although the project is still in its early stages, Finkbeiner reported that 53 genomes have already been processed and one candidate modifier identified—a proteasome variant. One of the biggest challenges, noted Finkbeiner, was to obtain samples from the unaffected parents of HD individuals, obtain sufficient multi-generational samples (which enhances the power of the search and reduces sequencing errors). As noted above, the Indiana roster was instrumental for overcoming these difficulties. Also, Finkbeiner pointed out that the NIH grant supporting the project is written so that additional individuals and families can be included in the study and the plan is to increase the number and quality of samples.

New technologies

Looking ahead, Finkbeiner hopes to extend his studies beyond the search for modifiers of age-of-onset to include modifiers of other HD phenotypes. A collaborative project with Google using machine learning algorithms to link the rich clinical datasets with the large set of cellular phenotypes Finkbeiner's team can track, should help accomplish this goal. In addition, this approach could reveal new cellular markers that could help stratify patient populations for clinical trials. As previously stated, most current clinical trials are comprised of participants with a wide range of phenotypes which can obscure positive results in a responsive subpopulation. Moreover, new technologies to modify genes of interest, such as Sangamo's zinc finger proteins, should allow the researchers to rapidly test candidate modifiers, expediting the path from hit to clinical trial.

Finkbeiner also described a new technology that promises to help deepen the understanding of HD cellular and molecular pathogenesis and suggest new therapeutic targets. The technology, developed by Nader Pourmand, allows the extraction and transcriptome analysis of tiny volumes of cytoplasm from single cells. A nanopipette is used for voltage-controlled aspiration of 15 femtomoles of cytoplasm and subsequent sequencing of its RNA with a method that requires less than 500 pg of total RNA. Finkbeiner is now collaborating with Pourmand to identify cellular pathways disrupted in HD and identify correlations between particular HD-associated alterations and a cell's transcriptional state. And because the collection of these nano-biopsies does not affect cell viability, cells can be monitored longitudinally using Finkbeiner's robotic microscopy system, so that transcriptional states can be correlated with phenotypic outcomes in individual cells.

Concluding thoughts

Participants were enthusiastic about the future. Although many challenges remain, the tools and resources available to the HD research community continue to grow and become more powerful. More importantly, the community itself keeps expanding and generating more ideas. The young investigators who were attending a Hereditary Disease Foundation workshop for the first time, including Ben Deverman and Gwen Owens, were

enthusiastic about the opportunity they had to attend the meeting. They found the workshop not only informational, but inspirational. It is the foundation's hope that, with their help, the future of HD will soon be changed.