HD2016: The Milton Wexler Celebration of Life

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Report prepared by Leora Fox, Ph.D.

Introduction

The 2016 Milton Wexler Celebration of Life brought together nearly 300 participants to discuss current research focused on developing treatments for Huntington's Disease (HD) and related disorders. Established researchers and collaborators new to the field shared the opportunity to speak with a family affected by HD, and heard about an unprecedented clinical trial of HD gene silencing. They discussed a host of novel techniques exploring genome editing as therapy, and participated in new sessions on DNA repair mechanisms and systems biology. The scientists present shared their most recent unpublished data, from the formation of mutant proteins in a test tube, to studies of animal behavior and large-scale human genetics. A wealth of new techniques, theories, and treatments in the pipeline contributed to an atmosphere of collaboration and excitement that will continue to motivate participants towards discovery.

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Part I: Clinical Aspects of Huntington's disease

To begin the meeting, Nancy Wexler expressed her excitement about the light-years of progress the HD community has made in the past half-century. She pointed to the numerous clinical choices available to today's patients compared to fifty years ago, and remarked on the recent burst of interest from the pharmaceutical industry. She encouraged the scientists present to speak their minds, thanking everyone for their continued bravery and willingness to share unpublished work, and adding that her father, Milton Wexler, would have been overjoyed.

Living with HD: family perspectives

Dr. Diana Rosas introduced the meeting's participants to Chris and his wife Marguerite, who are battling Chris's HD. Chris met Marguerite after the birth of her three children, which he described as a blessing, since he wanted kids, but did not want to risk passing on the HD gene. The couple described the early signs that clued them into the development of Chris's HD, which was diagnosed in 2007. Chris described eye movements, growing weakness in his fingers, and

difficulty with handyman tasks, while Marguerite noted slight changes in Chris's memory and reactions to everyday stresses. After 25 years working in sales, his difficulty with organization and applying new information at work had become increasingly stressful, so he retired amongst very supportive workmates.

Chris has struggled with bouts of weight loss, insomnia and irritability, which have been improved somewhat by medications. He talked about his increasing difficulty with eating, drinking, and getting dressed, and mentioned scary and frequent falls resulting from sudden movements and imbalance. However, when asked by a researcher what single symptom Chris would choose to fix, he responded, "memory." Dr. Rosas noted that he is not on medications to control chorea, but is managing well with help from physical and occupational therapists that help him strategize around his changing speech and movements. A failed test in a driving simulator helped him to make the decision not to get behind the wheel anymore, a push that Marguerite felt she could not make as a spouse. She noted the challenges faced by spouses and caretakers, particularly the difficulty of dealing with the family's diverse coping responses to the disease. Even among supportive friends and relatives, she explained that the pressure to exude positivity can sometimes negate the reality of the experience. She recalled anxious outbursts and close calls, and mentioned that a new symptom or a change in Chris can be accompanied, each time, by a sense of loss.

Nevertheless, Chris and Marguerite described their day-to-day life and their outlook on HD with humor, determination, and fierce positivity that inspired the meeting's participants. They described how modern technology like an iPad photo app has helped Chris to jog his memory, and how structuring his days with lists and volunteer work has helped him to keep active and maintain good sleep patterns. He participates in clinical trials, which included four years on creatine, and gives blood regularly for research purposes. Chris stressed that he wants to support future research for a cure in any way possible: "they can have my brain when I pass." It was a frank and inspiring conversation for the scientists present.

<u>George Huntington's legacy</u>

Public activism and patient involvement in medical research, as exemplified by Chris and Marguerite, has risen steadily in the past several decades. Two hundred years ago, HD was known only within isolated regions, such as the Long Island community where George Huntington practiced medicine. Alice Wexler presented a historical snapshot of the era, explaining the novelty of Huntington's 1872 description of the disease that took on his name. She laid out the circumstances under which this report, written by an inexperienced 22-year-old medical student, had a lasting impact on medicine and laid the foundation for research and advocacy.

Wexler emphasized that the novelty of Huntington's report lay in his description of the emotional toll of HD, and his perception of the inheritance pattern. Huntington's writing was informed by his father and grandfather's practices, centrally located within a community of Long Island families who were viscerally affected by the toll of HD. There was also regional interest in heredity due to a history of livestock breeding. In particular, he recognized, in a pre-Mendelian scientific atmosphere, that if a child did not inherit the disease, the chain of genetics

was broken. Importantly, his description, unlike that of other physicians of his time, emphasized how the disease might stop, rather than continue.

Huntington's seminal report helped to crystallize hereditary chorea as a distinct clinical entity and to give it attention from doctors. With more patients being seen in hospitals, increasing interest in the brain, and the introduction of neurology research journals, HD got a lot of attention in the late 19th and early 20th century. Recognized as a hereditary disease, it unfortunately became fodder for the arguments of the eugenics movement, and from the 1910s to 1960s clinicians recommended that those at risk be sterilized. However, in 1968, the formation of the HDF led to a landmark event shaping the research and policy agenda: a series of public hearings at which families with HD could testify about their struggles with the disease.

The identity of HD has shifted over time, and is slowly evolving away from stigma, secrecy, and shame. George Huntington was able to grasp both the outward-facing and hidden aspects of HD symptomology, alongside the social and emotional dimensions of the disorder. He incorporated everything into a concise and accurate description of a disease with accuracy and empathy, which foreshadowed the importance of medical research as a community pursuit.

<u>Development of gene-silencing therapy for neurodegenerative disease</u>

Fast-forward one hundred fifty years, and the HD community is in the midst of its first clinical trial of a drug that targets the genetic source of the disease. HD is caused by an expansion of more than 40 repeats of the nucleotides CAG within the *IT15* gene, which we usually refer to as the HD gene. The DNA gets transcribed into RNA, which is translated into the mutant huntingtin protein, widely believed to be toxic and dysfunctional. The treatment, created by Ionis Pharmaceuticals, uses a form of *gene silencing* called an antisense oligonucleotide (ASO). This is a small sequence of nucleotides designed to stick tightly to mutant huntingtin RNA so that production of the toxic protein can be blocked, lowering the amount present in brain cells. Similar targeted drugs that aim to lower levels of abnormal genes and proteins are in clinical development for a variety of neurodegenerative disorders in addition to HD.

Don Cleveland reviewed the preclinical work that led ultimately to the trial of the huntingtin ASO in humans. His lab pioneered the ASO approach in mouse models of amyotrophic lateral sclerosis (ALS) and later HD. Cleveland described the original experiments showing that BACHD and YAC128 model mice can recover from movement abnormalities and brain pathology when the HD gene is silenced with an ASO (Kordasiewicz et al., 2012). Importantly, the treatment can be given intermittently – even 4 to 9 months after a dose, mouse behavior is improved. In primates dosed through the spinal cord (similar to how humans will be dosed), the ASO penetrated to many areas of the brain, and has been well-tolerated. This result led to the concept of the "huntingtin holiday" – a respite from mutant huntingtin providing sustained benefit to the brain.

Only a decade ago his findings were met with great skepticism and disparaged as untenable for the purpose of human medicine. Today, enthusiasm from researchers and pharmaceutical companies in a variety of fields has led to even better designs for ASOs that are in early stage human clinical trials for ALS and HD.

Clinical trial of the huntingtin-lowering ASO

Sarah Tabrizi provided an exciting update on Ionis's huntingtin-lowering trial, and reemphasized the successful testing of the HD drug in mice, dogs, primates, and pigs. She explained that ASO treatment has many advantages: they are diffusible, stable, reversible, and they have a long half-life so infrequent dosing is possible. They do not cross the blood-brain barrier and must be delivered by intrathecal injection, via a needle inserted into the spine, but Tabrizi stressed that this is a safe and common procedure. The intrathecally delivered ASO does not penetrate perfectly to the striatum, which is particularly vulnerable in HD. Nevertheless, work from William Yang's laboratory suggests that the cortex, which would receive higher dosing of the ASO, may be even more important to target (Wang et al., 2014).

Ionis partnered with Roche to run the current Phase I clinical trial, in which the primary goal is to test safety and tolerability. Secondary and exploratory goals are to characterize how long the drug remains in the human brain and CSF, and to understand the pharmacodynamic effect of multiple doses. There are 36 individuals involved in the trial with sites in the UK, Germany, and Canada. There is a 3 to 1 ratio of active drug versus placebo, and the trial uses an ascending dose design, with 4, 8, 8, and 16 patients given increasing doses. This means a small initial group will get a low dose, and if this is well-tolerated, the next group will receive a higher dose, and so on. Eligible patients are diagnosed with early manifest HD, are between the ages of 25 and 65, live less than 4 hours away from a study center, and are accompanied by a study partner. They receive the injection every four months, and on study visits they undergo a variety of bloodwork and clinical assessments. Another exciting aspect of the trial is that Ionis has partnered with academic scientists to use the latest imaging and modeling technology to understand how the disease and the treatment affects the connections and structural integrity of the brain. In particular, a new technique called NODDI, a type of advanced MRI, is being used to map gray and white matter with high resolution.

So far, there have been no safety concerns associated with the drug injection, and researchers and clinicians are hopeful for a successful safety trial. Ionis has had recent success with an ASO of comparable basic structure, in the treatment of spinal muscular atrophy (SMA), a genetic disorder that leads to muscular weakness and death in infants. An August 1st announcement reported that this drug had met its primary endpoint mid-way through a controlled trial, meaning that it was appropriate to stop the trial and allow all participants to receive the ASO. The next step for the SMA ASO is to apply to FDA for drug approval. This gives us great hope for the ASO designed to treat HD.

Gene therapies and ASOs in development for ALS

In addition to studying HD, Don Cleveland's laboratory is developing potential treatments for ALS, a disorder that affects motor neurons, leading to extreme muscle weakness and death in mid-life. There are now over 160 known mutations that cause or heighten the risk of ALS, notably within an antioxidant gene called SOD1. Similar to our understanding of mutant huntingtin's role in HD, the disease is not primarily caused by losing normal function of SOD1, but by the mutated form gaining a toxic function. As with HD, the overarching strategy is to block the mutant SOD1 gene at its source, before the toxic protein is made. Lowering mutant

SOD1 with an ASO has strong protective effects, doubling lifespan in mouse models of ALS (Smith et al., 2006). The Cleveland group is adapting other genetic strategies, like viral delivery through the spinal cord, to silence SOD1 in primates, and preliminary results indicate that this approach can also knock down the gene widely and safely in the brain.

A second common ALS and dementia-causing mutation is a hexanucleotide repeat (GGGGCC) in a gene called C9ORF72, but it remains unclear why this mutation leads to disease. Current avenues of research in the Cleveland lab revolve around a possible decrease in the amount of normal C9ORF72 RNA (haploinsufficiency), or a toxic function of expanded RNA or protein inside the nucleus. They have recently published a genetic strategy in mice for lowering the expanded form, similar to their approach in HD and SOD1-caused ALS (Jiang et al., 2016). Based on their findings, ASOs may be a viable strategy for treating ALS due to mutation in C9ORF72.

Delivery of gene therapy for HD, ataxias, and childhood genetic disorders

Throughout the HDF meeting, a common theme in the discussion of challenges to gene therapeutics was delivery and brain distribution. Mutant huntingtin is present in every cell of the brain and body, but current methods of viral gene therapy based drug delivery can reduce its levels at best by 50-60%, highlighting an urgent need to explore more efficient delivery methods. Beverly Davidson's group uses a variety of small and large animal models to evaluate delivery and efficacy of gene therapies.

A main focus of Davidson's recent work is Spinocerebellar ataxia type I (SCA1). Like HD, SCA1 is a fatal inherited CAG repeat disorder where the expansion occurs in a protein called ataxin-1. This leads to cell loss in the cerebellum, pons, and brainstem, causing progressive difficulty with balance, coordination, and gait, with eventual inability to speak and swallow leading to death within 10-15 years. Both mutant huntingtin and mutant ataxin-1 protein contain a long stretch of the amino acid glutamine, represented by the letter Q, which is why HD and SCA are often referred to as polyglutamine or poly-Q diseases. In 2004, The Davidson group showed that even a 20% reduction in mutant ataxin-1 could have positive clinical consequences (Xia et al., 2004), and they have since been working on improved delivery strategies to more broadly treat affected areas of the brain.

One delivery strategy uses an adeno-associated virus (AAV) to deposit genetic material that can silence mutant SCA genes. A single injection into the cerebellum or deep cerebellar nuclei of SCA1 and SCA7 mouse models successfully spread the therapy and prevented the onset of cell loss and movement phenotypes (Keiser et al., 2014). This approach was later extended by postdoctoral scientist Megan Kaiser to study safety and delivery in the cerebellum of monkeys called rhesus macaques. They were able to achieve 30-50% gene suppression of ataxin-1 throughout a large proportion of the cerebellum without causing side effects in the monkeys (Keiser et al., 2015). Dosing experiments in SCA1 mutant mice have begun to reveal that higher doses promote augmented behavioral improvements, both preventatively and after disease had developed. In parallel, Harry Orr's lab is working on ASO-based strategies to target mutant ataxin-1, in partnership with Ionis Pharmaceuticals. They have identified one ASO that had significant benefits for survival and motor behavior in mice after just a single injection into the ventricle, and they are now optimizing the timing of delivery. Data from the Davidson and

Orr labs will be used to guide pharmacological and toxicity testing in primates and eventually humans.

A second focus of the Davidson lab is a family of recessively inherited neurological diseases known as lysosomal storage diseases. She shared work on late infantile neuronal ceroid lipofuscinosis, or LINCL. Kids with this disorder develop seizures, progressive blindness, and developmental delays, leading to death at around 10 years old. The disease is due to deficiency of an enzyme called TPP-I. Upping production of TPP-1 in just a small percentage of cultured cells can help the enzyme spread to neighboring ones (Sands and Davidson, 2006). It's also possible to genetically correct TPP-1 in ependymal cells that line the brain's ventricles, so the enzyme is circulated throughout the affected brain via the CSF (Liu et al., 2005). Currently, Davidson's group is testing this strategy in a daschund model of LINCL, and they have found that AAV delivery to ependymal cells can increase the amount of TPP-1 all over the brain and spinal cord (Katz et al., 2015). Recently they discovered that the animals' disease phenotypes, abnormal reflexes, cognition, and survival are significantly improved by treatment with the virus. This ongoing study will shape the future of treatment of LINCL, and its principles can be applied to the challenge of delivery in HD and other neurological disorders.

Additional therapies in the clinical pipeline

During Tabrizi's presentation on the IONIS ASO, one participant raised the point that these types of therapy, even if successful, would be prohibitively expensive for many people suffering from HD worldwide. Such ethical considerations, addressed by several speakers throughout the meeting, highlight the need for further development of small molecule oral therapies that might have similar effects. To this end, Blair Leavitt reported on four current clinical trials, on behalf of the Huntington Study Group, a collaborative global organization that aims to advance knowledge about HD and bring treatments to affected families.

FIRST-HD/ARC-HD is a trial by Auspex and Teva of deuterated tetrabenazine. Tetrabenazine was the first FDA-approved drug for treatment of chorea in HD. The deuterated form promotes a stronger bond with carbon, which serves to lengthen the half-life, permitting more convenient dosing and potentially reducing side effects. This trial had 90 participants with symptomatic HD, half of whom received a placebo control. Deuterated tetrabenazine was very well tolerated and resulted in a 20% improvement in chorea score compared to placebo. In an unprecedented demonstration of a treatment's ability to improve perception of chorea, patients also self-reported benefits of the drug. In comparison with regular tetrabenazine, it looks so far as though switching patients to the deuterated form, and ramping up dosage, may provide additional benefits. A report was published in JAMA in July (Geschwind and Paras, 2016).

PRIDE-HD is a trial of Pridopidine being carried out by Teva. Pridopidine is a putative dopamine stabilizer, also modulating glutamate and NMDA transmission. It may additionally confer protective effects for nerve cells, acting to balance communicative networks in brain areas controlling movement and mood. Leavitt explained that pridopidine has shown only modest benefits for HD in previous clinical trials, but these studies were not properly designed to test motor improvements. The current twelve-month trial is testing four different dosing levels plus placebo in 400 patients worldwide, mainly to determine movement outcomes. This

study is close to completion, with data analysis pending, and ongoing studies (called Open-PRIDE and Open-Hart) are designed for longer-term safety evaluation of pridopidine.

LEGATO-HD, also being pursued by Teva, is a trial of Laquinimod. This drug primarily targets inflammation, and has stimulated neuroprotective outcomes in patients with multiple sclerosis. During HD pathogenesis, there is an increase in NFkB, a complex that controls immune and inflammatory responses in addition to cellular processes like development, growth, and cell death. Laquinimod acts on these pathways within the brain's immune support cells, microglia and astrocytes, but the mechanism of its action is not exactly known. Enrollment for the trial is ongoing, and the goal is 360 patients dosed for 12 months, with two different dosages plus placebo. An additional high dose was dropped because of heart-related safety concerns. The primary aim is to improve motor score, but they are tracking many additional exploratory outcomes via brain imaging.

SIGNAL, a trial of VX15 being conducted by Vaccinex, aims to delay onset or slow HD progression in late prodromal (at-risk) and early manifest HD patients. This is the first double-blind placebo controlled trial of a biological molecule for HD. VX15 inhibits SEMA4D, which regulates cellular traffic in neurons and may activate a damaging inflammatory response during the progression of HD. Blocking SEMA4D is expected to correct "leakiness" of the blood-brain barrier, and may also help oligodendrocyte precursor cells (OGCs) to rebuild myelin sheaths, the conductive cushioning around neurons that allows them to communicate efficiently. The study will have an adaptive design with 84 at-risk or early symptomatic HD patients in two cohorts, receiving IV infusions of VX15 or placebo. So far, the first cohort of 48 patients has been recruited, and participants will undergo cognitive, motor, and brain imaging assessments.

Leavitt expressed excitement about the number of new trials, successful recruitment and retention of patients, and the fact that new drugs are being developed specifically based on what we have learned about HD, rather than trying things off the shelf.

Mapping patterns of atrophy in HD

With a wealth of brain imaging data emerging from clinical and observational studies like PREDICT-HD, TRACK-HD, and IMAGE-HD, there are increasing opportunities for analysis of brain networks. HD exerts its most potent effects on the cortex and striatum, and the damage is linked to problems with cognitive, emotional, and motor function. Govinda Poudel is using human imaging data to understand how the loss of connective pathways correlates with dysfunction in HD and determines the spread of disease through the brain. Work from TRACK–HD shows that MRI changes can be detected long before clinical symptomology, and that highly connected regions are implicated in dysfunction (McColgan et al., 2015). Poudel has used complex statistical modeling of imaging data to compare symptomatic HD patients, gene carriers, and unaffected individuals. His work has revealed that a brain area's functional proximity to disease regions can predict the severity of atrophy. In other words, if a healthy brain area communicates closely with a diseased area, the healthy area becomes dysfunctional more rapidly. The more closely connected a cortical area was with the striatum, an HD epicenter, the more susceptible it was to cell death and loss of connectivity. Because our understanding of anatomical progression of the disorder is limited, further analysis will be required to expand this work into other brain regions affected in HD.

Biomarkers

Two years ago, at the HD2014 meeting, there was emphasis on the need for additional and improved biomarkers, measurable substances in the body that can indicate disease progression or signal the effectiveness of treatment. This year, we have real therapies in the clinic, and Dr. Ed Wild reported on the standardization of sample collection and the hunt for biomarkers in cerebrospinal fluid (CSF). He pointed out that the ideal biomarker should be directly downstream of what a therapy aims to alter. In the case of HD gene silencing, intended to lower mutant huntingtin levels in the nervous system, it could be highly informative to measure the amount of mutant huntingtin protein circulating in the CSF during treatment. He explained that CSF is likely to be a more accurate source of biomarkers than blood, because it bathes the brain and spinal cord and is continuously turned over. Additionally, CSF levels of mutant huntingtin are predictive of motor score and cognitive performance in HD patients.

Wild reported on an assay he developed in the Tabrizi lab that can be used to measure mutant huntingtin levels in the CSF with single-molecule sensitivity (Byrne et al., 2017), which will be featured in the anti-huntingtin ASO clinical trial. He also described an international CSF collection initiative called HDClarity. The initiative aims to ensure that CSF is collected, handled, and processed similarly worldwide. This type of reproducible methodology opens many avenues for the study of patient samples.

Miriam Moscovitch-Lopatin, working in the laboratory of Steven Hersch, introduced a novel soluble oligomeric mutant huntingtin biomarker for which they developed and validated an assay. Monomeric mutant and endogenous huntingtin assays were developed and validated as well, to detect these three huntingtin species, believed to differ in toxicity. The assays were enabled by a monoclonal rabbit anti-huntingtin antibody, developed in collaboration with Marian DiFiglia and Kim Kegel-Gleason. These three assays were deployed in the PRE-CELL longitudinal study, led by Vicki Wheelock, and showed that blood and CSF levels of each are correlated consistently between patients, potentially allowing for blood testing, which is less invasive than a spinal tap for CSF. This alternate approach is being validated in this observational clinical trial, and may be further developed as biomarkers for emerging therapies.

Part II: Understanding the normal function of huntingtin

Although reducing mutant huntingtin is a major aim of current genetic therapies in the pipeline, we have a limited understanding of how the mutation causes the protein to wreak havoc as it performs its usual functions. To this end, several invited talks at the meeting showcased work aimed at elucidating the normal role of huntingtin. Basic research on how different regions and features of the huntingtin protein contribute to a growing array of cellular processes serves as an important basis for understanding how the mutation leads to the disease. In biological research, understanding the function of protein or protein segment often involves removing it and observing what occurs when it is gone, or creating structural additions that affect its levels, recycling, or movement.

Huntingtin in cortical development

Without huntingtin protein, developing mouse embryos cannot survive. Sandrine Humbert's work focuses on huntingtin's role within neural stem cells and progenitor cells, the developmental precursors of cells in the nervous system. She hypothesizes that the HD mutation causes brain development to occur abnormally in individuals with HD, exerting long-term effects that extend into middle age. Humbert summarized our knowledge about the structure and function of normal huntingtin protein in embryogenesis and adulthood. She explained that huntingtin is a large protein that has more than 100 distinct conformations *in vitro*, and 120 binding partners. It is critical for microtubule-based transport, cell division, and the generation of cilia. During adulthood, knocking out the huntingtin gene leads to neurodegeneration in mice, suggesting that in HD there may be effects of losing the normal function of huntingtin due to the CAG repeat mutation.

During the birth of new neurons, known as *neurogenesis*, progenitor cells first divide symmetrically, then asymmetrically. Then they stop dividing, develop distinct bipolar shapes, and migrate along radial glia. To understand the role of huntingtin in dividing progenitor cells and post-mitotic (non-dividing) cells, Humbert's group used genetic strategies to eliminate huntingtin from brain cells during early stages of embryonic development. In the absence of huntingtin, asymmetric division of the progenitor cells occurred too early, leading to premature birth of new neurons (Godin et al., 2010). Mutant huntingtin also leads to this type of premature cell division (Molina-Calavita et al., 2014). Removing huntingtin from post-mitotic cells delayed the migration of new neurons to their appropriate layers in the cortex, and also impaired the development of distinct morphology and directional reaching (Barnat et al., 2017). Mutant huntingtin also impairs neuronal migration.

Humbert's group found that this developmental impairment occurs in the absence of huntingtin due to improper recycling of an adhesion molecule called N-cadherin, which is important for the attachment of migratory cells along their routes. The recycling of N-cadherin is controlled by a regulatory protein called RAB11. Huntingtin usually helps to maintain and energize RAB11, so when it is either missing or mutated, RAB11 can't properly maintain the recycling of N-cadherin. Consequently, overexpressing RAB11 in embryos lacking huntingtin restored proper migration of developing brain cells. Early developmental removal of huntingtin affected cells even as the mice approached adulthood, causing a migration delay associated with changes in the shape of neurons' receptive arms (dendritic morphology). This work suggests that the effects of HD may begin as early as gestation, which could have long-lasting implications later in life.

Another developmental process gone awry in HD was presented by Daniel Wilton, a postdoctoral researcher in Beth Stevens' lab. During development of the brain, numerous connections form between neurons that must be pruned away, retain only the strongest and most relevant synapses. This is accomplished in part by immune cells called microglia that engulf elements of synapses to weaken and destroy unwanted connections. Wilton hypothesizes that this process may be occurring abnormally during adulthood in diseases like HD, leading to weaker synaptic connections between cells of the cortex and the striatum. He has demonstrated that the connective pathway between striatum and cortex is vulnerable to synaptic loss in HD, and that synapses in diseased brain are frequently engulfed by microglia.

Studying BACHD and zq175 mice, Wilton has found that striatal synapses express high levels of molecular "eat me" signals called C1q and C3, which signal microglia to destroy the connections. Ongoing work shows that HD mice lacking the C1q tag are protected from synaptic loss. In collaboration with Annexon biosciences, the Stevens lab is testing whether an antibody against C1q can slow disease progression in HD models.

Daniel Paredes from the Lieber Institute for Brain Development presented an additional viewpoint on how developmental pathways might affect cellular survival in HD. He is studying a growth factor important for the health of neurons and maintenance of synapses called BDNF, which is decreased and dysregulated in HD. BDNF mediates signaling by binding tyrosine receptor kinase B (TrkB) to exert its effects. Working with cultured neurons from mouse hippocampus and rat models of BDNF deficiency, Paredes is looking for non-invasive ways to restore TrkB signaling. He is studying a precursor to the sleep hormone melatonin called N-acetylserotonin (NAS), which can activate TrkB pathways. Preliminarily, treatment of rats with NAS had positive effects on the survival of inhibitory neurons. Paredes is working on understanding a possible mechanism for this finding, and would like to determine whether NAS treatment could delay onset or perhaps help to counter the sleeping impairments that are common to both patients and animal models of HD.

Huntingtin's role in aging

Gene silencing has increasingly promising therapeutic potential, but some of the current approaches would also target normal huntingtin. Whereas researchers have uncovered essential roles for huntingtin during development, we don't fully understand the consequences of removing normal huntingtin during adulthood, such as might occur during a genetic therapy. Xiao-Jiang Li uses a *conditional knockout* strategy in mice to remove huntingtin at multiple points of development, in various cell types of the body and brain, and at different stages of adult life. A good analogy is that of drawing dotted lines around the whole or partial huntingtin gene, then supplying only certain types of cells at certain times with the genetic "scissors" to chop the gene out.

Recent work in Li's lab has investigated the role of huntingtin in aging, by deleting huntingtin from every cell throughout the entire body and brain at 2, 4, or 8 months old in mice. These ages are comparable to adolescence, reproductive peak, and middle age. They found that the younger they deleted huntingtin, the fewer mice survived, pointing to its continuous importance in early life. However, none of these age groups showed deficits related to neurodegeneration, cell death, or behavior. Rather than neurological complications, the mice had peripheral and abdominal problems, the root of which was pancreatic failure. Li's group has identified a gene called SPINK1 which interacts with huntingtin to regulate enzymes in the pancreas. When huntingtin is removed in young adult animals, there is heightened enzymatic activity in pancreatic cells called ascites, leading to intestinal inflammation and eventually death.

Deleting huntingtin only in neurons did not have drastic effects on survival, except for a small percentage of the youngest mice, and deleting huntingtin only in the cortex and striatum was not fatal for any of the mice. These results suggest that huntingtin's role varies in importance during aging, but removing the normal form does not have major effects on the

brain, which is good news for gene therapy. Nevertheless, Li suggests that because of peripheral effects, targeting particular brain areas might be safer than whole-body approaches.

Huntingtin's first exon

The first exon of the HD gene contains the polyQ expansion that causes HD as well as two other important flanking regions, the N17 region (denoted as N) and the polyproline region (denoted as R). To better understand the function of these regions of huntingtin, Scott Zeitlin's team performs targeted genetic manipulations in mice, then characterizes their behavior and biochemistry. Zeitin shared a large body of published and preliminary data from several mouse models in which portions of normal exon-1 huntingtin had been homozygously deleted, or heterozygously deleted opposite mutant huntingtin on the other allele. This included deletion of the N17 region (N, amino acids 2-17), the polyQ and polyproline regions (P/Q, amino acids 18-56), or all three (NPQ).

In each of these models, the Zeitlin lab examined survival rates during and after development, measured behavioral performance with locomotor and memory tasks, and examined functional and neuropathological phenotypes. Overall, embryonic development and survival was not significantly affected by deleting the N, P, or Q regions of huntingtin, and behavioral effects were mild and fluctuated with age. For example, young mice with N17 or PQ deletions first outperformed their wild type littermates, then developed deficits as they aged. A similar pattern was observed in mice with one mutant huntingtin allele (Q140) and one wild-type allele with exon-1 deletions, promoting enhanced learning capability during adolescence followed by impaired locomotor activity during old age, 18 months for a mouse.

Deletion of the polyproline region on the same allele as the 140Q expansion produced the most pronounced effect, delaying aggregation and rescuing neuropathological phenotypes like glial activation, striatal cell stress, and accumulation of lipofuscin. This manipulation also restored changes in gene expression within disease-relevant pathways, such as levels of the antioxidant protein Gpx6. Using their huntingtin mutation/deletion mouse lines, Zeitlin's team is currently working on a screen to examine which proteins preferentially interact with mutant versus wild type huntingtin.

Judith Frydman presented an additional perspective on how the polyQ flanking regions could control the appearance, behavior, and toxicity of huntingtin. In the human brain and in various model systems, mutant huntingtin forms clumps called aggregates. Frydman's lab manipulates huntingtin *in vitro* to determine how flanking region deletions and mutations result in different rates and types of aggregate formation. Another focus is a chaperone protein called TRiC that helps to refold proteins during disease-related stress to brain cells. She pointed out that our conception of aggregate building is usually linear, going from monomers (single huntingtin proteins), to oligomers (several huntingtin proteins stuck together), to large stably structured aggregates (many huntingtin proteins in a clump with other proteins). However, she argues that aggregate formation and disassembly may be better represented by a more dynamic conception of the energy it takes to build and dismantle different types of structures. Current work in the Frydman lab is focused on huntingtin oligomers with different flanking region mutations. They showed that removal of the polyproline (R) region results in fewer and more transient oligomers, while removal of the N17 (N) region leads to extra and more stable

oligomers, suggesting that these regions play opposing roles in the formation of the aggregates that characterize HD.

Adding these different conformations of mutant huntingtin to slices from rat brain revealed that removing either flanking region (N or R) is toxic alone, but removing both is not. Frydman believes that this reveals a disconnect in the way we study aggregation and toxicity. She presented preliminary evidence suggesting that the R region of huntingtin is required to help expose the N region for proper interaction with protein targets. Current and future experiments in her lab aim to determine how different forms of huntingtin interact with protein homeostasis networks, and how this may affect toxicity.

Huntingtin in autophagy and immune pathways

Joan Steffan presented her work on huntingtin as a potential mediator of autophagy, a self-digestion process that allows cells to break down their contents and recycle damaged or deviant machinery. During autophagy, cellular materials are engulfed by structures called autophagosomes, then trafficked to lysosomes which contain recycling enzymes. In HD and other neurodegenerative disorders, clumps of potentially toxic proteins can be removed via autophagy. Steffan's previous research suggests that huntingtin is not just a substrate of this pathway, but a participant in selective autophagy, serving as a scaffold that allows selective clearance of autophagic cargos by the lysosome (Ochaba et al., 2014).

More recently Steffan has shown that immune pathways are intricately connected with huntingtin accumulation and clearance. A major current focus is the IKK complex, the origin of a molecular cascade crucial for immune, inflammatory, and cell death responses. The IKK complex has also been implicated in the induction of autophagy (Criollo et al., 2010) and as a direct interactor with mutant huntingtin (Khoshnan et al., 2004). Steffan's previous work showed that IKK increases phosphorylation of serines 13 and 16 of huntingtin and can activate its clearance by autophagy (Thompson et al., 2009). She is now working with an R6/1 HD mouse model in which IKK-beta can be removed during adulthood. So far they have found that IKK-beta knockout during HD leads to lower levels of phosphorylated huntingtin and affects the expression of autophagy genes. IKK-beta knockout also accelerates behavioral phenotypes in the R6/1. Her work reveals a significant amount of overlap between huntingtin's presence as both a scaffold and a substrate for protein clearance systems.

Given Steffan's work on huntingtin as a potential mediator of selective autophagy, Scott Zeitlin also examined autophagy in many of his huntingtin deletion mutants. With deletion of segments of normal exon-1 huntingtin, no major changes occurred in autophagic function. However, in cultured cortical and striatal neurons from N17 deletion/Q140 mice, they observed an increase in autophagic flux. This suggests that a simultaneous stress source is required to impact a potential autophagic function of these regions of huntingtin. Conversely, Xiao-Xiang Li observed no overall changes in basal autophagy in his adult huntingtin knockouts. However, since huntingtin is proposed to be involved in more selective forms of autophagy, this topic merits further research.

Part III: DNA repair in HD

The 2016 HDF meeting marked the first year in which a session was devoted to the discussion of DNA repair in the context of Huntington's Disease. DNA is in constant use in order to modify and control biological messaging and the construction of cellular building blocks. DNA damage occurs continually in all human cells due to environmental factors like sunlight, lifestyle factors like smoking, and normal cellular processes like metabolism. DNA damage can rise during aging and disease due to increasing oxidative stress. There are numerous types of lesions that can affect DNA, and various teams of proteins that counteract this constant wear and tear. During this session, Ray Truant proposed a novel biological role for huntingtin in the repair of damaged DNA. David Housman and Leslie Jones commented on emerging data from humans suggesting that DNA repair mechanisms can modify the age of onset of HD symptoms. Agata Smogorzewska, new to brain research, contributed expertise about a specific DNA repair gene called FAN1.

A role for huntingtin in DNA repair

As described in Part II, there is growing evidence for huntingtin protein's involvement in a myriad of essential biological functions, including development, aging, maintenance of normal behavior, and autophagy. Ray Truant's lab is exploring the possibility that huntingtin protein may also respond to cellular stress like DNA lesions. Huntingtin's properties and location within cells is influenced by molecular tags called post-translational modifications. Recent work in the lab has shown that a single post-translational modification to the N17 region of huntingtin exon-1 (the addition of a phosphate group, known as phosphorylation) sends huntingtin to the nucleus after oxidative stress (DiGiovanni et al., 2016). Truant and colleagues introduced different chemical agents and point mutations that made huntingtin more likely to receive phosphorylation tags that promoted entry into the nucleus.

A recent innovation in the Truant lab uses a "chromobody" system in human HD cell lines that allows for visualization of huntingtin movement in live cells. They are currently trying to determine what types of oxidative stress could trigger the addition of nuclear entry tags to huntingtin protein, and why huntingtin would need to enter the nucleus in response to oxidative stress. Postdoctoral scientist Tam Maiuri has gathered evidence that huntingtin is called to the nucleus to help fix damaged DNA, and that the summons comes from a DNA repair gene called ataxia telangiectasia mutated (ATM). Huntingtin co-localized with ATM at sites of DNA damage, and it interacts with a variety of other proteins known to repair DNA lesions. They hypothesize that it acts like a scaffold in the nucleus to support DNA repair. The mutant form of huntingtin is able to locate to the right sites within the nucleus but can't properly perform its DNA repair function, leading to extra DNA damage in cells with the HD mutation. This theory is supported by work in William Yang's lab showing that decreasing ATM had benefits for HD mice (Lu et al., 2014). This work has raised additional questions about the types of molecular machinery that might regulate huntingtin's response to oxidative stress and link it to DNA repair.

DNA repair genes modify age of onset in human HD

The extreme variation in the age of onset of Huntington's disease is a great mystery that has begun to be broached via large-scale genetic research on HD gene carriers. Several recent studies of this nature have fortified the potential link between HD and DNA damage, suggesting that a number of DNA repair genes can act as *genetic modifiers* of HD. This term refers to genes with one-letter genetic variations called single nucleotide polymorphisms (SNPs, pronounced "snips") that are usually inconsequential. However, in a person with the HD mutation, these slight changes can hasten or delay the expected onset of movement symptoms. The difference between the *actual* age that symptoms occur, and the age they would be *expected* to occur based on that person's CAG repeat length is known as the *residual* age of onset.

As a member of the Genetic Modifiers of Huntington's Disease consortium (GeM-HD), Leslie Jones spoke to the importance of identifying relevant modifier genes, so that we can pursue therapeutic approaches within the biological pathways most likely to have real influence on symptomatic onset. SNPs leading to large residuals, both positive and negative, are worthy of further investigation. Jones shared research published by the GEM-HD consortium (Genetic Modifiers of Huntington's Disease, 2015) and unpublished data revealing genetic modifiers of HD within loci associated with DNA repair pathways, mitochondrial events, and redox reactions.

Jones is now heading a study in patients with an array of polyglutamine disorders, analyzing data from a cohort of 1500 patients with HD, spinocerebellar ataxias (SCA), dentatorubral-pallidoluysian atrophy (DRPLA), and spinobulbar muscular atrophy (SBMA). Many of the SNPs relevant to age of onset of HD can similarly modify symptomatic onset in other CAG repeat disorders (Bettencourt et al., 2016). Jones emphasized that many neurological diseases involve faulty DNA repair, causing synaptic and neuronal defects that lead to dysfunction and death. Some of the identified modifiers have been implicated in cell-specific pathways, a step towards understanding why certain types of brain cells are affected early in HD. There is also an association between particular types of DNA lesions and CAG repeat expansion in somatic cells, suggesting that this link will be an important avenue for future genetic studies. Jones is hoping to expand her research in two main ways: first, amassing more samples, and second, looking at whole genomes or exomes to pinpoint more specifically which genetic sequences are most relevant to age of onset.

David Housman spoke about similar efforts to identify genetic modifiers of HD within a different patient population. The HD gene was originally identified through studying a large interrelated population of individuals on the shores of Lake Maracaibo in Venezuela. Through the efforts of Nancy Wexler, scientists helped to obtain NIH funding and clinicians went for 23 years to the region, collecting data from thousands of people including 20,000 neurological exams and 8000 cognitive exams. In 2008, Wexler and Housman led a genetic linkage study that correlated altered age of onset with changes in specific regions of the genome (Gayan et al., 2008). To understand which SNPs modify age of onset in humans, Housman is now delving further into these genetic loci and integrating the clinical data with complete genomic sequences from the same patients. Wexler and Housman are looking for genes that cause both earlier and later age of onset. So far they have full genomic sequences of more than 500 people, and they have identified several top candidates. One gene, called Alfy, will be discussed further in Part V. Two of these genes, called FAN1 and RRM2B, are relevant to DNA repair. A

SNP in FAN1 caused HD symptoms to onset on average 6 years earlier than expected, and this finding was reinforced by data from the GEM-HD consortium, suggesting that DNA repair genes may play an important role in HD.

A role for FANCD2/FANCI-Associated Nuclease 1 (FAN1) in HD

Given that unbiased research within multiple human datasets pointed to FAN1 as a potential modifier of HD, organizers of the HDF 2016 meeting invited Agata Smogorzewska to share her expertise on FAN1. Smogorzewska studies the repair of intercrosslink (ICL) damage – a frequent but abnormal situation where the two complimentary strands of DNA are improperly connected, impeding transcription and DNA replication. Persistence of unrepaired ICLs can lead to diseases of development, cancer susceptibility, and organ failure. Cells without FAN1 are extremely sensitive to DNA damage, and in humans, a genetic lack of FAN1 leads to a rare disease of the kidney, which can be modeled in mice (Thongthip et al., 2016).

In the context of HD, Smogorzewska is working in collaboration with David Housman to determine what types of damaging agents and DNA lesions may be most detrimental to adult brain cells when FAN1 function is missing or compromised. She introduced two early theories about the intersection of DNA repair and HD: first, a mutation in FAN1 might lead to increased somatic expansion of CAG repeats. This would mean that in individual dividing cells or organs in an affected person's body, the CAG repeat expansion in huntingtin could become even longer. Alternatively, FAN1 could be involved in the maintenance of DNA in non-dividing brain cells. If it were exceptionally important in the vulnerable cells of the striatum, this could explain an earlier HD onset when FAN1 is compromised. These hypotheses are currently being tested via collaborations with HD researchers, and an additional HDF workshop was organized to promote further discussion of FAN1's role as a genetic modifier.

Part IV: Protein Homeostasis in HD

The balance between the genesis and breakdown of cellular proteins is known as *proteostasis*, a portmanteau of protein and homeostasis. In a variety of neurodegenerative disorders, mutant and misfolded proteins build up within cells, which is evidence that the proteostatic balance may be compromised. Several conference participants are studying how different cell types experience proteostatic imbalance and respond to the challenge of misfolding and aggregation during aging and disease.

Proteostasis Collapse in aging and HD

Rick Morimoto presented recent evidence that heat shock proteins (HSPs) and J-proteins, families of chaperone molecules that aid protein assembly and disassembly, can function to disaggregate and resolubilize toxic mutant forms of proteins like huntingtin (Nillegoda et al., 2015). When beset by mutant proteins over the course of years, this machinery has difficulty coping, and to compound this dysfunction, mutant huntingtin itself may interfere with many aspects of protein quality control in various compartments of cells.

Morimoto suggests that if we could slightly rectify the cellular imbalance, this might have large overall effects on symptomology.

Morimoto's lab works with *c. elegans* worms expressing polyQ expanded proteins that aggregate as they age. Using RNA screens, they previously identified specific transcription factors that influence responses to stress, altering the course of aggregation and affecting the lifespan of the worms (Labbadia and Morimoto, 2015). These include a wide variety of genes that perform protein synthesis, folding, and degradation. Recent work in the Morimoto lab suggests that in humans, many of these genes decline in expression during both HD and normal aging. The remarkable overlap in HD and aging suggests that the age-related decline in proteostasis capacity is exacerbated by disease.

These observations led members of Morimoto's lab to ask in *c. elegans* at what age and rate the expression profile begins to change, and whether these expression changes are specifically programmed. They found that misfolding begins to occur within early adulthood, corresponding to a reduction in HSPs. This makes the worms much more vulnerable to stress as they age, with a noticeable change pre- and post-fertility. Within hours of reproducing, they become susceptible to stress-induced death. This response is controlled by a specific demethylase that "closes" the chromatin (the packaging surrounding DNA), making it much more difficult for the cell to respond to protein misfolding. This switch is signaled by adult stem cells in the worm, and when it is repressed, the aging worms are much more stress-resistant. Similarly, a dramatic shift in stress resistance and aggregation can be achieved by making slight modulations in the expression of genes related to mitochondrial health and protein homeostasis. Current work in the lab is focused on a large screen to identify small molecules with subtle stress-resistance effects in cells, brain slices, and ultimately *in vivo*.

Targeting protein homeostasis networks with drugs

Jason Gestwicki at UCSF also focuses on how molecular chaperones can influence the course of disease. Huntingtin itself is folded, refolded, disaggregated, and degraded by a diverse array of cellular machinery. Mutant huntingtin's expanded CAG repeats increase the likelihood of misfolding events that require action by chaperones. When HD, aging, and oxidative stress cause the demand on these chaperones to approach capacity, the proteostatic balance verges on collapse. Gestwicki outlined four important focal points in proteostasis research in HD and other polyglutamine disorders: (1) Using genetic screens to identify the right pathways to target, (2) validating these targets using genetic tools, (3) discovering the mechanism of the target pathway through biochemistry and cell biology, and (4) testing discoveries in animal models. To rebalance protein homeostasis in HD, Gestwicki's overall goal is to identify the most important chaperones and redesign them "smarter," increasing the robustness of the system that protects against protein aggregation.

Gestwicki's lab, like Morimoto's, has also focused on heat shock proteins (HSPs), but with a targeted structural biology approach. Previous research from his group has defined the structure of HSP70 and has elucidated its protective role in cell and fly models of HD and other neurodegenerative disorders (Pratt et al., 2015). Gestwicki's team uses chemical biology techniques to design and test specific inhibitors of chaperone complexes to increase the system's capacity for refolding. They have screened close to a million molecules and narrowed

their search down to a few that successfully inhibit interactions between HSP70 and nucleotide exchange factor (NEF) or HSP40 (J protein), shifting the homeostatic balance so that less aggregation occurs.

In collaboration with Andy Lieberman at the University of Michigan, Gestwicki is working on HSP70's role in a CAG repeat disorder called Kennedy's disease, or spinobulbar muscular atrophy (SBMA). The inherited polyglutamine expansion occurs in the androgen receptor (AR) protein, leading to hormonal and muscular problems primarily in men. HSP70 is involved in a "triage" decision to either ignore a misfolded AR, or signal the cell's machinery to remove it. Gestwicki and colleagues have found three AR binding sites in the N-terminus of HSP70. Their goal is to change the decision-making process of HSP70, so that it binds for longer to the mutant AR and signals for its breakdown. Postdoctoral scientists Xiaokai Li and Hao Shao are performing painstaking chemical analysis to customize a drug that blocks a nucleotide exchange factor (NEF) that normally uncouples AR and HSP70. Ultimately, this drug will help HSP70 attach for longer to the AR, shifting the chaperone system to favor turnover of defunct, toxic androgen receptor. They have also identified a co-chaperone, HSP70-interacting protein (Hip) which has similar stabilizing effects as their drug. Hip overexpression speeds turnover of androgen receptor and ameliorates a muscular phenotype in expanded AR flies. Recently, they have been testing Hip overexpression and analogs of their drug in an SBMA mouse model, and preliminarily, they can reduce aggregated AR in the muscle tissue. Gestwicki stressed that this is not a clinical drug candidate, but a proof-of-concept experiment. Nevertheless, this careful and design-based approach to intervention in proteostasis has yielded promising results.

Autophagy's role in proteostasis in HD

While chaperone systems act to unfold and refold proteins and make molecular decisions about their fate, degradation machinery serves to remove and recycle damaged structures, equally impacting proteostasis. One such pathway is macroautophagy, in which autophagosomes engulf materials in the cytoplasm and transport the contents to lysosomes for recycling. Ai Yamamoto studies selective macroautophagy, focusing on the autophagy-linked FYVE domain protein (Alfy), which acts to scaffold mutant huntingtin aggregates and other large abnormal structures into the autophagy machinery. Her previous work showed that brain cells are capable of removing mutant huntingtin aggregates (Yamamoto et al., 2000), and implicated macroautophagy in this process (Yamamoto et al., 2006). In 2010, her lab found that huntingtin can be tightly enveloped by autophagosomes through a selective form of aggregate macroautophagy, and that this process requires Alfy (Filimonenko et al., 2010).

Yamamoto explained that Alfy is enormous and highly conserved, and it interacts with the machinery that drives autophagy. Without Alfy, aggregated forms of huntingtin and other disease-relevant structures and large complexes cannot clear in cells. Because it is very specific to the removal of aggregates, the Yamamoto lab is now trying to understand its function in vivo by genetically depleting Alfy in mouse models of HD. In the BACHD mouse model, originally developed by William Yang, lowering Alfy leads to more aggregates, worsened behavior, and accelerated brain pathology. They showed that this was due to a lack of aggregate clearance using an adult knockout of Alfy combined with silencing of the huntingtin gene. Furthermore, cellular experiments using photo-convertible dyes showed that Alfy promotes turnover of

existing, stable huntingtin aggregates, rather than turning over growing structures or preventing the formation of new aggregates (Eenjes et al., 2016). In short, without Alfy, neurons cannot clear accumulated mutant huntingtin deposits, and this accelerates the onset of behavior and pathology, suggesting that removing aggregates could promote recovery. This piece of information may be key in the pursuit of more specific therapies to increase selective autophagy and remove the most toxic types of mutant huntingtin aggregates.

In an exciting development, Alfy has also been implicated as a genetic modifier of HD in humans, which was discussed further by David Housman. Yamamoto's evidence that Alfy could modify HD onset in mice led Housman's group to check whether SNPs in Alfy could change age of onset in humans. Wexler and Housman are sequencing the DNA of the Venezuelan families to identify modifiers of age of onset. In fact, one rare SNP in Alfy delayed age of onset by an average of a decade. In one family of eleven siblings with very late HD onset, three had a delay of onset by as much as 23 years, and within their DNA sequences was this rare variant of Alfy. It is by far the most protective variant they have discovered so far. This is an exciting find which will drive future work towards therapies that might target this region of Alfy or its corresponding molecular pathways - we are looking hard to uncover its mysteries and turn them into treatments.

The toxic conformer of huntingtin

As mentioned by both Rick Morimoto and Judith Frydman, huntingtin is found in a variety of conformations, resulting in myriad states that require different levels of energy to maintain (Hartl et al., 2011). Many presenters touched upon the idea that the toxicity of huntingtin is dependent upon its conformation. However, there are a wealth of theories in the ongoing debate about which form of mutant huntingtin contributes most to disease progression. Early research on this topic showed that fragments of exon-1 huntingtin with polyQ expansions would self-assemble into aggregates *in vitro*, and that certain forms of huntingtin could "seed" the rapid formation of larger aggregates (Scherzinger et al., 1997; Scherzinger et al., 1999). Twenty years after this observation, Erich Wanker's working hypothesis is that stable, amyloidogenic, seeding-competent mutant HTT protein conformers drive disease development in HD patients and model systems. In other words, the most pathogenic forms of huntingtin are those that can trap other proteins and cause large aggregates to grow.

To pursue this hypothesis, the Wanker lab has created fluorescence-based assays using techniques called FRET and FRASE in which they can analyze the seeding ability of different types of huntingtin fibrils. Using mouse and human tissue, they have correlated this property with toxicity and disease severity. They found that even mutant huntingtin from pre-symptomatic HD mice is able to induce seeding, such as tissue from 1-day-old R6/2 mice with no aggregation detectable by immunohistochemistry. Seeding-competent mutant huntingtin is often found in biochemical fractions that are usually thought of as "soluble" and thus non-toxic, challenging the traditional definition of an aggregate. Furthermore, human tissue shows seeding ability, more so in brain areas that are highly affected by HD.

To look at whether these seeds truly confer toxicity, Wanker's group used fly models to test fragments of aggregates with different CAG repeat lengths and different seeding

competency. They find that disease severity correlates with CAG repeat length and with the amount of time the HD fragment genes were turned on, with longer repeats or prolonged expression leading to more detergent-insoluble aggregates and shorter lifespans. Surprisingly, even short exposures to mutant huntingtin protein significantly decreased the lifespan of the flies. The seeding abilities, rather than the biochemical solubility of the aggregates, correlated strongly with toxicity. Furthermore, a short period of overexpression of chaperone HSP70 can decrease seeding and extend the flies' lifespan. These findings provide a novel perspective on the meaning of a harmful aggregated structure, and suggests that prevention of seeding could be a therapeutic strategy.

Post-translational modifications influence the processing of huntingtin

The movement and processing of huntingtin can be influenced by the addition of protein tags known as post-translational modifications. In addition to the tag described by Ray Truant that results in nuclear translocation, Joan Steffan and Steve Finkbeiner discussed modifications that impact huntingtin removal via protein degradation. Steffan's previous work has shown that huntingtin phosphorylated at serines 13 and 16 is more efficiently processed by autophagy, and this can reduce toxicity in model systems (Thompson et al., 2009). This is further supported by *in vivo* work in collaboration with William Yang's lab indicating that S13 and S16 phosphorylation can impact the abundance of huntingtin aggregates and reduce disease severity (Gu et al., 2009).

Work from Steve Finkbeiner's group suggests that phosphorylation of serine 421 (S421) helps to remove a toxic huntingtin conformer, detectable by an antibody called 4H7H7 (Miller et al., 2011; Peters-Libeu et al., 2012). Recently, they showed that inducing or genetically mimicking this modification (S421D) mitigated neuropathology and behavioral deficits in BACHD mice and preferentially reduced the toxic conformer. They went on to show that huntingtin S421D is more readily removed by a clearance pathway known as the ubiquitin proteasome system (UPS). This particular type of protein modification is likely to occur on other types of poly-glutamine expanded proteins that are relevant to diseases like SCA and SBMA. The modified serines discussed by Steffan, Finkbeiner, and Truant may be interesting targets to consider in the design of therapeutics.

RAN translation in HD

The excess CAG repeats within the HD gene are translated into a long stretch of the amino acid glutamine within the mutant huntingtin protein, which is widely believed to be a major source of toxicity in the pathogenesis of HD. However, long CAG repeats can sometimes be interpreted by the cell's machinery as stretches of AGC, GCA, or their opposing-strand complements. This leads to the formation of several other expanded protein peptides, a phenomenon known as repeat-associated non-ATG (RAN) translation. These RAN translation products can contain repeats of the amino acids glutamine, serine, alanine, leucine, and cysteine, forming additional aggregation-prone peptides that challenge our conception of toxicity in HD.

RAN translation was discovered and characterized in the laboratory of Laura Ranum, where molecular tags revealed unexpected aggregates of expanded CUG repeat RNA in a cellular model of SCA8 (Zu et al., 2011). The phenomenon has also been described in other diseases, like myotonic dystrophy and ALS, in which the pathogenic mutations lie in non-coding genetic material (DNA that is not normally used to make proteins). Ranum's recent work has focused on identifying toxic RAN translation products that accumulate in HD brain cells.

Postdoctoral scientist Monica Banez-Coronel designed distinctive antibodies to identify RAN protein products (Banez-Coronel et al., 2015). In cell models, mice, and in the brains of HD patients, these peptides often formed harmful, insoluble clumps, similar to the huntingtin protein. In humans, these RAN protein aggregates appeared in white matter bundles of the communicative highways of the striatum and cortex, which are highly vulnerable in HD. The RAN proteins showed cellular and anatomical distribution patterns that were distinct from that of mutant huntingtin, even accumulating in the cerebellum, which is rarely marked by huntingtin deposition. They were often found in proximity to activated microglia and apoptotic cascade proteins, suggesting that RAN products are associated with immune responses and cell death. The longer the CAG repeat stretch, the more abundant and punctate were the RAN protein aggregates. Transient transfection of the RAN peptides into neuronal cell lines was toxic independent of whether the cells expressed the HD gene. Overall, Ranum cautioned that we need to step back and re-examine our notion of whether huntingtin protein is the only source of pathogenesis in HD.

Part V: Systems biology in HD

Science is in the midst of a revolutionary period of discovery through genomics and systems biology; researchers are able to integrate experimental and computational approaches to analyze large-scale data sets derived from thousands of samples in humans or model organisms. These methods, sometimes referred to as "omics," can incorporate entire genome sequences (genomics), encompass RNA expression (transcriptomics), and determine protein or lipid changes (proteomics and lipidomics) as HD progresses. The 2016 HDF meeting devoted an entire session to discussions of systems biology, in which researchers presented statistics-driven discoveries in large datasets from ataxia models, HD mice, human stem cells, *c elegans* worms, and *drosophila* fruit flies.

<u>Systems biology approach to SCA1</u>

Over the past twenty years, Harry Orr has published seminal work modeling SCA1 in a variety of systems, studying the toxic effects of the mutant ataxin-1 protein. Orr's research in mice and cells has defined an important role for the location and post-translational modification of mutant ataxin-1 in pathogenesis. The original transgenic SCA1 model recapitulated motor deficits and dysfunction of cerebellar Purkinje cells (Burright et al., 1995; Clark et al., 1997). Further work from Orr and collaborators also revealed key aspects of ataxin-1 dysfunction that led to pathogenesis: phosphorylation at S776, entry into the nucleus and interaction with a transcription factor called capicua (cic). Recent unpublished work in targeted

genetic mouse models has revealed that the interaction of cic and Ataxin-1 in the nucleus is required to cause both behavioral deficits and Purkinje cell pathology.

To identify pathways associated specifically with cerebellar Purkinje cell death, the lab recently undertook a comparative RNAseq analysis during aging in two types of SCA1 models. One model expresses ataxin-1 with a pathogenic mutation (82Q), and shows ataxia and cerebellar pathology. A second model expresses ataxin-1 with a non-pathogenic CAG repeat length (30Q) and an S776D mutation mimicking phosphorylation. This model develops the motor signs of ataxia, but no cerebellar pathology. By comparing widespread gene expression (RNA levels) in these models over time at 5, 12, and 28 weeks old, Orr's group can track the molecular markers reflecting progression of cerebellar pathology, versus protection against cerebellar pathology (Ingram et al., 2016).

Put simply, their statistical approach (as well as several other presenters in the session) involves grouping sets of genes into related "modules," based on each gene's expression level, function, or location. Then they map the changes within each module over time to determine which correlate most highly with onset and progression of ataxia. While there are likely a myriad of cellular pathways contributing to toxicity, Orr suggests that a module of downregulated genes in Purkinje cells may reflect disease progression, while a module of upregulated genes in cerebellar cortex may reflect a compensatory response. To make this distinction, they took a subset of these genes and compared the up-and-down-regulation between their mouse models with Purkinje cell death versus protection. In the protected model, they found upregulation of cholosystekinin (cck), a regulatory signaling molecule and feeding hormone. Correspondingly, genetically deleting cck abolished the protection. In the mice with Purkinje cell death, boosting cck with a small molecule agonist had protective effects on behavior and pathology.

Integrated genetic and systems approaches to study HD

HD usually onsets during adulthood, but it has been difficult to determine the exact relationship between CAG repeat length and aging as drivers of pathogenesis. To explore this question, William Yang is using RNAseq techniques to compare aging under normal circumstances to aging during the course of HD. His lab generates and interprets transcriptomics and proteomics data, looking at how levels of RNA and proteins change over time in mouse models and in humans. After disease strikes, we observe that expression data shifts – but do these changes occur suddenly, or gradually? Which brain regions are most affected, and which networks change similarly during aging and HD?

In collaboration with Steve Horvath, Peter Langfelder, and Giovanni Coppola at UCLA, Yang recently published on an allelic series of knock-in HD mouse models with CAG repeat lengths of 20 (nonpathogenic), 80, 92, 111, 140, and 175, examining RNA-seq and proteomics data at 2, 6, and 10 months of age in multiple brain regions (Langfelder et al., 2016). They found that increases or decreases in RNA and protein expression scaled with both age and length of the CAG repeat, occurring continuously over the lifetime of the animals. This was especially true in the striatum, and less so in the cortex or the liver. Recent work has focused on validating the most significant 800 genes in the Q175 mice at 2, 3, 4, 5, and 6 months old, using a sensitive gene expression technology called Nanostring. This unpublished study, also validated in

humans, has confirmed that there are gradual gene expression changes occurring between early and middle age.

Using network analyses similar to those described by Harry Orr, Yang's analyses clustered data into modules according to a gene's location and function. Expression changes that were dependent upon CAG repeat length were most commonly found in striatal modules, suggesting that this is where the earliest disease-related changes occur. Significant expression changes occurred in genes related to striatal identity – in other words, the neurons most vulnerable in HD appeared to lose the properties that make them unique. Yang proposes that mutant huntingtin impairs the ability of medium spiny neurons to maintain their identity and distinguish them from other types of brain cells as they age. Without this identity, the cells are much more likely to develop dysregulated apoptotic signaling and succumb to cell death.

Expanding their analysis of HD mice to include the cortex, striatum, hippocampus, and cerebellum, the Yang lab has recently identified genes that change in opposite directions in the striatum versus the cerebellum, which may be interesting candidates to validate as potential drug targets. They are also examining the brain and liver of wild type mice to understand what types of expression changes occur during normal aging. In the striatum, they see changes in many of the same markers that are downregulated or upregulated in HD, but this positive correlation does not extend to other brain regions. Consequently, Yang hypothesizes that part of the molecular pathogenesis of HD is actually accelerated regional aging.

Understanding dynamics and impact of molecular reprogramming in HD

Though HD symptoms don't normally manifest until adulthood, mutant huntingtin likely exerts negative effects during development, reshaping how the brain responds to stress. Christian Neri studies how abnormal mechanisms persist from development through the onset of symptoms. His lab uses statistical analyses to integrate microarray data across different model species, including human stem cells, c elegans worms, mice, and postmortem human tissue, grouped into early, intermediate, and late phases of HD pathogenesis. Their statistical modules cluster genes according to their involvement in defined cellular pathways, many of which shift in expression levels as the disease state progressively worsens. Neri's team observed the greatest downward shifts in genes associated with synapses, and interestingly, genes associated with inter-cellular communicative vesicles called extracellular exosomes. These vesicles have a variety of functions, from conveying signals between cells, to cargo transport, to maintaining homeostasis in synapses. They have validated their findings experimentally in striatal cells from mice as well as human neuronal stem cells, showing that there are fewer small extracellular vesicles originating from endosomes in HD cells.

Neri described a second type of network analysis that is allowing the lab to compare the molecular changes that occur in slow versus fast-developing mouse models at different stages of symptomatology. In this case, they clustered expression changes by cell type to see whether vulnerable cells in HD experience specific patterns of change. Reflective of William Yang's findings, striatal cells in HD mice acquire a non-striatal RNA and protein signature: their genetic identity appears to devolve during aging and as CAG repeat expansion lengthens. Furthermore, striatal changes begin to occur even before the mice begin showing behavioral symptoms.

The top genes underlying these major shifts in the striatum are involved in synaptic homeostasis, voltage-gated ion channels, and stress response pathways. Neri's group has validated several genes individually through immunofluorescent staining of R6/2 mouse brain, such as the increase in a transcription factor called ROR-alpha. Also altered in their analyses was FOXO3, a transcription factor controlling stress pathways which is protective in HD mouse models (Parker et al., 2005; Parker et al., 2012). Their systems biology data has converged to show that a number of genes regulated by FOXO3 are altered in a poly-Q and time-dependent manner over the course of HD. They are now working with human neural stem cells to better understand how FOXO3 loses its normal targets and gains abnormal ones as it governs the stress response during development and HD. Neri's ultimate goal is to understand how developmental reprogramming of vesicular traffic, striatal identity, and stress governs the progression of HD.

Novel statistical approaches to omics data

While "omics" approaches have been tremendously informative towards basic biology, they have rarely impacted medicine, as data concerning DNA, RNA, and proteins does not always agree. Furthermore, many of the targets identified in these studies do not lie in known or adaptable pathways. Ernest Fraenkel described new statistical approaches to address this challenge, searching for consistency within many types of data to determine the best pathways to target. His lab uses novel computational strategies to identify genes that show reliable directional alterations as HD progresses in humans and models. By combining the mathematics of ordinal regression with gene expression data, they can perform unbiased comparative analyses of proteomics, transcriptomics, lipidomics, and other network changes. Essentially this serves to dramatically narrow things down to a few new genes of interest, which can be validated with computational analyses and benchside experiments in cellular models of HD.

Importantly, the Fraenkel lab's unbiased and biased statistical methods have identified many of the same genes, which they have gone on to validate at the bench. For example, one major hit that emerged from this process is sphingosine-1-phosphate (S1P), a protein involved in signaling pathways that promote cell survival. From large-scale statistical analysis, to identifying its partners in a biological pathway, to using a chemical inhibitor to protect cells expressing mutant huntingtin, this gene has proven a consistent target that emerged from their new method.

The lab is using other types of large scale unbiased analyses to understand which lipid binding proteins and post-translational modifications are most relevant to the cellular signaling changes observed in HD. Postdoctoral scientist Leila Pirhaji developed a novel modeling technique called PIUMet that aligns data related to DNA, RNA, proteins, and lipids to identify pathways highly affected in HD. The heart of her algorithm serves to simplify "crazy network hairballs" down to a core set of connected biological pathways. Fraenkel noted the emergence of sphingosine pathways from this analysis, further validating findings related to S1P. At the moment, these analyses are focused on the HD brain as a whole; it will be important to determine whether these networks differ across brain areas or cell types.

Molecular pathogenesis: disease drivers versus compensatory mechanisms

Both Yang and Neri spoke to the idea of a shift in striatal cell identity in HD, while Ernest Fraenkel questioned whether this change is pathogenic or compensatory. Is HD causing vulnerable striatal cells to lose their identity, or do striatal cells adapt to resemble a less vulnerable type of cell? Juan Botas is approaching the question of pathogenic versus compensatory gene expression changes by combining omics data with a real functional output. His lab crosses HD *drosophila* (fruit flies) with knockouts or overexpressors of hundreds of candidate genes identified in screens, then uses a simple climbing test to determine if the genetic perturbation improved or exacerbated the HD phenotype.

The Botas lab has designed a large automated behavioral system to test two types of HD flies, one with a mild phenotype, and one which exhibits a progressive, late-onset phenotype with impaired climbing to the top of a test tube. In collaboration with researchers like William Yang, or members of the GeM-HD consortium, Botas aims to validate transcriptomic and proteomic data from mice and humans by testing how individual genes of interest affect HD fly behavior. They mimicked the under- or over-expression of around 300 genes that show significant expression changes in mouse or human brain. For example, CACNA1B is downregulated in HD patients and mice, and when they lowered its levels in HD flies, their climbing deficits improved, suggesting that the under-expression in humans was compensatory. ACTN2 is also downregulated in the brain of HD patients, but when they lowered it in HD flies, the climbing phenotype worsened, suggesting that this change is pathogenic. Many of the genes that showed the strongest effects operate within networks controlling synaptic vesicle release, calcium signaling, and energy metabolism within the cell. Collaborator Boxun Lu is similarly validating these genes by evaluating the survival of stem-cell-derived neurons. Together these types of experiments provide functional readouts to test whether gene expression changes in HD are pathogenic or compensatory, better characterizing potential foci for therapeutic intervention.

Integration of omics, stem cells, and mouse models

Transcriptional changes are an early feature of HD, and these changes can be mediated by the state of chromatin, a regulatory structure that encases and compacts DNA depending upon which genetic material needs to be accessed or replicated. The term *epigenetics* refers to a change that affects expression but not the sequence of a gene. For example, transcription can also be controlled by a process known as methylation, where a molecular tag is added to part of the DNA complex, essentially preventing normal interpretation of the genes.

Leslie Thompson works with induced pluripotent stem cells (iPSCs), derived from human fibroblasts, that can be reprogrammed into a variety of cell types. In collaboration with a large international consortium of scientists studying iPS cells from patients with HD (Consortium, 2012), she has shown that the dysregulation of many genes and proteins can be modeled in patient-derived cells grown in a dish. Sergey Akimov also reported the consortium's findings on a variety of gene expression and metabolic changes in striatal cells derived from HD patient iPSCs. Examples of network changes in both RNA and protein levels are decreases in energy

levels, synaptic vesicle pathways, mitochondrial pathways, and oxidative phosphorylation. Akimov is following up with functional analysis of mitochondrial metabolism.

Thompson's lab investigates iPSC derived models through large-scale epigenetic and "omics" screens of DNA, RNA and proteins that are misregulated in HD. They combine their findings with patient data, functional cellular assays, and validation in animal models and computational systems. In 2013, Thompson and collaborators examined the striatum of R6/2 mice using a technique called ChIP-Seq to map the acquisition of detrimental epigenetic tags, and identified the regulatory enzymes responsible. They showed that H3K4 trimethylation is associated with dysregulated genes in HD (Vashishtha et al., 2013), and blocking it could protect neurons and promote the survival of HD flies. Statistical modeling has revealed that R6/2 mouse brain and iPSCs from HD patients show similar dysregulation patterns and altered epigenetic signatures, particularly with those genes involved in calcium signaling and developmental networks. There was an increase in REST, a master regulator of neuronal development, leading to dysregulated expression reflecting a delay in maturation.

A top candidate of recent interest is NeuroD1, a transcription factor essential for neurogenesis. NeuroD1 may be improperly regulated by mutant huntingtin. In neuronal iPSCs from HD patients, inducing NeuroD1 with a drug called Isx-9 rescued HD phenotypes like impaired neurite outgrowth and sensitivity to growth factor (BDNF) withdrawal. Steve Finkbeiner's lab showed similar results in HD cells with lower numbers of CAG repeats. Finally, Isx-9 improved cognition and rescued synaptic pathology in R6/2 mice, but did not improve motor behavior. Overall, Thompson hypothesizes that neurodevelopmental changes like maturation defects could have lasting effects on structural connectivity and adult neurogenesis. The lab continues to explore identification and drug delivery around genetic targets that remain consistent across model systems.

Jose Lucas also shared data validating a set of genes that emerged from large-scale analyses, called cytoplasmic polyadenylation element binding proteins (CPEBs). These are RNA binding proteins that promote the elongation of the polyadenylation (polyA) tail of mRNA to activate or repress translation. There are four related forms of CPEB in mammals, and CPEB4 targets many genes that are involved in Huntington's disease pathways (Ortiz-Zapater et al., 2011). Lucas is exploring the involvement of CPEBs in HD pathogenesis, and has validated gene expression analyses in mouse models of HD and other neurodegenerative disorders as well as in HD patient tissue. His lab's work has confirmed that CPEB1 is increased and CPEB4 is decreased in HD, and as a result, the polyalanine tail length of hundreds of transcripts is altered, impacting protein levels of the affected genes. Genetic overexpression of CPEB4 in the forebrain of HD mice normalized protein levels, and improved motor behavior and neuropathology.

Part VI: Novel methodologies and models

<u>Linking mice and humans</u>

Alongside the challenge of extracting relevant drug targets from large-scale data sets, it is difficult to predict whether preclinical data from cellular and animal models will scale up to real therapeutics in patients (Crook and Housman, 2011). How can we better understand the

relationship between the tests we perform on patients and those we use in animals? Steve Finkbeiner aims to address this challenge by linking patients and preclinical models through the design of similar experimental paradigms. For example, his lab has created a human virtual reality navigation test that simulates a type of spatial memory test in mice called a Morris water maze. Whereas the mice must navigate through opaque liquid to find a hidden platform, Alzheimer's patients use video controls to find treasures in a maze. Analyzing the results from both humans and mice will help to refine animal testing so that it is as analogous as possible to patient testing.

Studying patient iPSCs

A second way that Finkbeiner is hoping to combat the disconnect between preclinical and clinical data is to work with human iPS cells differentiated into a variety of brain cell types. The Finkbeiner lab has for many years employed and improved upon an automated single cell analysis system to develop "clinical trials in a dish." Automated microscopes and camera equipment allow them to follow the fate of every living cell in an experimental plate in response to different drugs or genetic manipulations. This approach has allowed them to make robust models out of human cells with genetic mutations for diseases like ALS, Parkinson's disease, and HD. An array of biosensors and analyses can then be used to follow phenotypes like survival, aggregation, synaptic structure, metabolism, and transcription levels for relevant genes. The automated technology has also helped to identify novel targets and small molecules that can ameliorate disease states in a dish. In particular, they have identified ways to induce the autophagy system to break down aggregates and promote survival in their cellular models of HD and ALS. Finkbeiner has recently partnered with computer scientists at Google to create smart image analysis systems that can identify structures and predict outcomes in cells based on visual information. He also suggests that their automated systems can be used as a platform to find or validate genetic modifiers of HD that would vary among individuals in large families with the HD gene.

Leslie Thompson is also using iPSC-derived cells to create a cellular model of the blood-brain barrier in HD, which protects neurons from substances in circulating blood. In collaboration with Dritan Agalliu at Columbia, Thompson's lab has shown that human HD-derived epithelial cells have an overactive response to injury and form an ineffective barrier, permitting entry of toxic substances that compromise their survival. Integrating omics screens with functional assays suggest that the cells have increased angiogenesis and impaired maturation. This new model could also potentially be used to evaluate transport of drugs across the BBB.

Modeling HD with directly converted patient neurons

Andrew Yoo is exploring an alternative strategy to study cells that come directly from patients. He works with small noncoding RNA molecules called microRNAs, which exert control over cell type during neurogenesis. Over the past several years he has developed a strategy to directly reprogram human fibroblasts into different types of neurons. This is different from an iPS cell strategy which first reverts cells to a pluripotent state; Yoo's strategy simply converts

cells from one type to another. Two microRNAs called miR9 and miR124 can be applied to fibroblasts to turn them into neuron-like cells called miNs (Yoo et al., 2011). By adding a cocktail of transcription factors they can be converted into very specific neuronal subtypes, including cortical neurons, spinal cord motor neurons, and striatal medium spiny neurons (Victor et al., 2014). The miN MSNs express many of the biological, morphological, and electrophysiological properties that define human striatal cells. Yoo's team can even implant these cells into the brain of a mouse, where they integrate into circuits and emit the proper inhibitory neurotransmitters. (Richner et al., 2015). Michael Levine has been exploring this implantation technique with human stem cells to rescue electrophysiological and behavioral deficits seen in R6/2 mice, and it may be interesting to use healthy MiNs to explore this strategy in HD mouse models.

Recently, members of Yoo's laboratory used the MiN strategy to create a novel model of the HD striatum, by converting patient fibroblasts into MSNs. After conversion, striatal cells with pathogenic CAG repeat lengths (40-50) showed EM48-positive aggregated huntingtin. They also saw decreased survival of HD cells compared to normal ones, as well as a DNA damage phenotype. Preliminarily, they have compared cells converted into MSNs and cortical neurons, and while they see aggregates in cortical cells, MSNs are much more vulnerable to death, as occurs in the HD brain. From studying the cells of children, adults, and elderly individuals, they have also found that the epigenetic changes acquired through a person's life are maintained in the converted miN cells, making them a robust model to study aging-related changes in both health and disease. Yoo's work represents a useful new tool for studying aging and HD in a dish, while retaining many of the features of the disorder.

Genome editing: opportunities for HD

Genome editing is the precise, controlled creation of genetic mutations for the purpose of research or therapeutics. CRISPR/Cas9 is one of many varieties of this technology, and it has been widely adopted due to its ease of application. It is lifted from a real biological pathway in bacteria; the basic idea is that the CRISPR sticks to and stabilizes a specific piece of DNA, while the cas9 nuclease, or cutting enzyme, makes a snip. In this way, an exact genetic sequence can be expanded, altered, or removed (Cox et al., 2015; Hsu et al., 2014). Matt Porteus explained that when the CRISPR/Cas9 system makes its cut, it activates the cell's internal repair mechanisms that either (1) stick the cut ends back together to achieve a deletion, or (2) paste in a new section of DNA. Researchers can design "donor DNA" segments that change a single letter, or add a new sequence to a specified location. Porteus illustrated examples of how his lab is using CRISPR/Cas9 to address real medical challenges, and proposed genome editing strategies that might someday be employed in Huntington's disease.

One donor DNA strategy in HD research reflects recent findings related to genetic modifiers. For example, CRISPR technology could be used to create a SNP in models that has been shown to delay age of onset in humans, to study its effects in model systems. Porteus's group has explored this strategy in the context of sickle cell anemia, a red blood cell disease caused by a single point mutation in human hemoglobin. They are using a novel viral strategy that allows the donor DNA and the CRISPR nuclease to enter cells without being recognized as foreign by the immune system. Porteus estimates that about 30% of the patient's red blood

cells with the mutation could be corrected outside the body using CRISPR, then given back to the patient. He is applying to begin a clinical trial of this approach to treat patients with sickle cell disease.

The lab is also exploring larger genetic replacements in relation to a rare immune disorder best known as "bubble boy disease." In HD, this replacement strategy could potentially be used on a much larger scale to actually replace the entirety of expanded exon-1 with normal exon 1 in cells with the HD mutation. Another speculative idea is to knock in protective or replacement genes in human neural stem cells in a dish, and then reimplant those cells into the brain. In mice, these cells can actually integrate into networks in particular brain areas. Porteus suggests that a "gene inside a gene" strategy could turn on a protective gene when an apoptotic one is activated, to prevent cell death. They are also exploring a deletion strategy targeting the N and proline flanking regions within exon-1 of mutant huntingtin, to remove the CAG repeats. Using two nucleases, they create pairs of DNA cuts that are rejoined by the cell's internal systems to remove the CAG repeats with up to 85% efficiency in vitro. He is interested in further developing this strategy in model systems in collaboration with HD researchers.

Porteus offered a checklist for genome editing strategies to promote discussion of current possibilities. Therapeutically, before we can employ genome editing for the treatment of HD, we need to understand what we should correct, what cell types to engineer, and how to deliver the therapy, which remains the largest challenge. He discussed some of the pros, cons, and challenges of editing outside or inside the body and mentioned two important considerations about nucleases: one, it can be dangerous if they are constantly active, and two, they could invoke an immune response. His group recently found that 90% of women have existing antibodies against Cas9, suggesting that we would not be able to use these strategies so easily in vivo.

Many of Porteus's proposed strategies for HD are extremely speculative ideas, and research will need to first determine which modifications would be ideal, let alone safe. Nevertheless, this talk stimulated an excellent discussion. Participants brought up the idea that when considering these approaches, we have to take into account the many brain areas and cell types affected by HD. Furthermore, we don't fully understand the potential off-target effects of CRISPR in human cells or how they would affect symptomology in humans. Porteus also brought up an important ethical consideration: given that even genetic diagnostics are inaccessible to many, we have to develop treatment strategies that are financially accessible to people all over the world.

Genome Editing Challenges

Feng Zhang's seminal work has focused on refining CRISPR to create even greater precision and efficiency, through the careful design of the accompanying Cas9 cutting enzyme. His techniques have brought about unprecedented ability to quickly create genetic variations, additions, or deletions and perform large-scale screens to determine the biological effects caused by loss or gain of function (Konermann et al., 2015; Shalem et al., 2014). Zhang discussed the major challenges to potential therapeutic applications of CRISP/Cas9: delivery and specificity. Enzymes like Cas9 are large molecules that must be packaged into viruses that can help access DNA, and they cannot reach every single cell. Furthermore, they can sometimes

cause unwanted genetic changes or induce dangerous immune responses. To overcome this challenge, Zhang is exploring smaller Cas9 enzymes and other nucleases and customizing their structure for maximum specificity and safety.

Recently, they showed that a smaller cas9 enzyme could provide successful targeting in human cell lines and *in vivo*, through an experiment to genetically reduce cholesterol in the mouse liver (Ran et al., 2015). They also engineered a new chemical structure based on the electric charges within the CRISPR-Cas9 complex, to create a more stable bond with target DNA and minimize the likelihood of off-target genetic damage (Slaymaker et al., 2016). Zhang described how his lab is using different versions of this improved gene editing technology to explore bacterial diversity, neuronal maturation, and therapeutics. By examining many bacterial subtypes, his lab has identified and characterized another cas9-like enzyme called Cpf1 that has the potential to be equally specific and effective (Zetsche et al., 2015). They continue to search for and identify candidate enzymes that may prove to be even more effective alongside CRISPR or novel gene editing systems. Finally, the Zhang lab has applied CRISPR/Cas9 technology to better understand the molecular identity of neurons in the developing brain, mapping changes in gene expression to different stages of neuronal maturity.

During the meeting, several other researchers described efforts to employ CRISPR/Cas9 in HD research. Zhang mentioned the strategy of exon-1 removal or shortening the CAG repeat tract in the HD gene to a non-pathogenic length. Don Cleveland is employing parallel CRISPR strategies in human cells, and has also achieved more specificity with fewer off-target effects. Bev Davidson's group showed that removal of exon-1 via CRISPR can be achieved in mice using viral delivery into the stratum, and they are currently exploring the behavioral implications in mouse models. Xiao-Jiang Li's laboratory is also using a viral CRISPR/Cas9 strategy to permanently knock out the CAG repeat mutation in the striatum of homozygous HD knock-in mice. Their preliminary results indicate that this has begun to reduce the amount of huntingtin aggregates, brain pathology, and motor deficits. For the HD field, this work represents a very exciting new use of a valuable genetic technology, and supports the idea that it will be possible to generate safe and effective genetic therapies.

Phenotype changes in HD sheep

Researchers most commonly model HD and other disorders in mice, but often the therapies that are effective in rodents do not translate to the human clinic. Jenny Morton discussed the importance of large animal models in the study of HD and shared an update on the HD sheep. These animals provide a means to study relevant biomarkers and therapeutic efficacy in the context of slowly-progressing pathology, and can help us with drug scaling and delivery in an era of real potential treatments. Another advantage is that blood samples can be collected continuously to look at circulating metabolites over a whole day, in the context of very controlled feeding and housing schedules.

The HD sheep model, studied so far up to about 5 years of age, expresses the human huntingtin cDNA at low levels throughout the whole brain, which is of a size and complexity comparable to human brain. They show aggregates in the cortex by three years old which increase over time, in addition to metabolic changes in the cerebellum and liver (Huntington's Disease Sheep Collaborative Research et al., 2013). So far, the HD sheep have no overt motor or

social deficits, but they do show abnormal sleeping patterns unless housed with normal sheep. Recently, Morton and colleagues examined metabolites in the blood of 5-year-old sheep, uncovering a very clear metabolic phenotype. The HD sheep showed dysregulation of the urea cycle, a waste breakdown process performed in the liver and kidneys, as well as changes in nitric oxide pathways that control cellular signaling. Examining groups of metabolites together can predict with high confidence which blood came from an HD sheep and which from an unaffected sheep, suggesting that groups of metabolites in blood could be explored as a biomarker for changes in HD. The team will continue to explore metabolic changes in younger and older animals.

Making use of the large sheep brain to understand how neuronal communication is disrupted in HD, Morton and her team are performing imaging, electrophysiology, and EEG recording during cognitive tasks. Using implanted tetrodes, they can measure the activity of individual sheep neurons in response to behaviors performed in real time. Because they can record from the same cell for up to weeks at a time, this could be a unique opportunity to study how neurons change over time in response to mutant huntingtin or to treatments. As the sheep age, collaborators are developing ways to test complex HD symptomology, such as cognitive and memory issues, executive function, apathy, difficulty swallowing, and compromised sleep patterns. With a variety of subjects after years in development, Morton invited participants to reach out if they were interested in validating behavioral, electrophysiological, or metabolic functions in the sheep model in the context of HD symptomatology and treatment.

Part VII: Electrophysiology

Neurons communicate through electrical impulses known as action potentials. While genetics and biochemical approaches are useful for understanding molecular changes in cells over the course of HD, it's equally important to investigate the electrophysiological properties of affected brain regions. Previously, electrophysiologists primarily studied HD by recording from single neurons in brain slices, or stimulating them to evoke currents. Today, the field has been revolutionized by optogenetics, a method of inserting receptors in the brain that can be controlled through light, essentially making investigators capable of controlling the response of individual cells or brain circuits. Several participants discussed electrophysiological findings in HD cells and mouse models, elucidating how brain circuitry and signaling contributes to HD.

<u>Altered functional microcircuits in Huntington's disease</u>

Michael Levine gave an overview of the circuitry within the basal ganglia, the region of the brain containing the striatum. The striatum, a major structure of the basal ganglia, contains two main circuits that coordinate movement: one pathway promotes voluntary movement, and one suppresses it. Put very simply, in HD, the death of cells that suppress movement causes symptoms of excess movement. Much of the focus of HD research has been on medium spiny neurons, because they comprise 95% of the striatum and are vulnerable to death in HD. However, striatal cells rarely die in mouse models, and other interconnected cell types may contribute to pathogenesis. For example, cholinergic interneurons make up about 2% of the cells in the striatum. Levine's laboratory has focused recently on two main types of

interneurons, persistent and low threshold spiking (PLTS) and fast spiking (FS) cells, which express somatostatin and parvalbumin, respectively. They found using optogenetics that in HD, dysregulated input from these cells leads to excess inhibition of MSNs but in differing ways (Cepeda et al., 2013).

The Levine lab is also studying the role of large cholinergic interneurons (LCIs) in HD pathogenesis. Compared to the ubiquitous spiny neurons, these cells are perserved during the course of HD, but Levine's laboratory has found that they are dysfunctional in R6/2 mice, exhibiting less regular firing due to an increase in spontaneous inhibitory inputs. Using optogenetic techniques to silence and activate different populations of neurons, Levine's team found that PLTS interneurons are responsible for over-inhibiting the LCIs. Functionally, this decreases acetylcholine output, which could explain the drop in acetylcholine levels in HD patient brains and in mouse models.

A second aim is to dissect the microcircuitry among spiny neurons within the direct and indirect pathways of the striatum, due to long-standing evidence in humans that spiny neurons within the indirect pathway are affected earlier in the course of the disease. Levine's team has recently shown that during HD pathogenesis, spiny neurons within the two pathways receive distinctive kinds of input and relay different dysregulated output. Josh Barry, a postdoctoral trainee in the laboratory, has recently shown that the direct and indirect pathways have differing electrophysiological effects on their targets in the substantia nigra (SNr) and globus pallidus (GPe), respectively. Activating the direct pathway generated a reduced amplitude response in the SNr, while activating the indirect pathway generated a response of increased duration in the GPe. This also affects the local connections amongst spiny neurons of the different pathways. Future research aims to understand the molecular mechanisms of these phenomena, such as loss of presynaptic fibers, or changes in neurotransmitter reuptake mechanisms.

A third current project, representing a new direction in the laboratory, uses calcium imaging through a cranial window to examine the activity of cortical microcircuits in behaving symptomatic HD mice. These studies are showing that there is reduced local calcium signaling or "bursting" and greater overall synchrony in the cortex of the R6/2. The conclusion is that there are complex alterations in circuits that affect how synaptic connections function, and these are unique to each brain region and subpopulation of neurons. Therefore, we should consider designing therapies specific to particular brain regions, circuits, and cell types.

Synaptic dysfunction in HD

The basis for communication between neurons is the release of chemicals called neurotransmitters from one cell to another across synapses. Most excitatory connections use the neurotransmitter glutamate. When the presynaptic cell fires an action potential, glutamate is released across the synapse onto channels called NMDA receptors. The NMDA receptors open and allow ions to flow through, making the postsynaptic cell more likely to fire. The strength of this relationship is the basis for learning and memory. However, it also underlies the phenomenon of "excitotoxicity," where prolonged overactive firing can cause cells to malfunction and die. This is thought to occur during the pathogenesis of Huntington's disease.

Lynn Raymond studies how glutamate excitotoxicity may preferentially affect the vulnerable neurons of the striatum and cortex.

Whether communicative versus toxic pathways are activated by NMDA receptors depends upon their location and their composition. Raymond explained that NMDA receptors at synapses promote strengthening of connections, while those outside of synapses (extrasynaptic) promote cell death pathways that can be toxic. In 2010, her group showed that YAC128 HD mice had more extrasynaptic NMDA receptors in the striatum, promoting cell death pathways. Recently, they found that this occurs as early as 4 weeks old. Using a combination of chemical and optogenetic strategies in brain slices and in vivo, they found that both cortical and thalamic inputs to the striatum contribute to increased extrasynaptic NMDA current (Kolodziejczyk and Raymond, 2016).

To look in real time at the timing of glutamate uptake and clearance from synapses, Raymond's team used a fluorescent reporter called iGluSnFR that would glow green when glutamate was attached to NMDA receptors. Surprisingly, glutamate did not linger in the synapses of R6/2 mice, but was cleared away faster, challenging other methodologies indicating impaired glutamate uptake in HD models (Parsons et al., 2016). Importantly, Raymond pointed out where her lab's techniques or conclusions conflicted with previous findings, and invited discussion about therapeutic strategies related to suppressing excitotoxicity. She also suggested that some of the genetic modifiers identified in recent human sequencing studies may contribute to the localization of NMDA receptors, thus preventing or hastening toxicity.

Synaptic store-operated Calcium entry and sigma1R as novel therapeutic targets for HD

Communicative connections between neurons are strengthened by the entry of calcium into the postsynaptic cell. Calcium ions are released from the endoplasmic reticulum (ER) through inositol triphosphate receptors (InsP3R). Previous work from Ilya Bezprozvanny's laboratory showed that mutant huntingtin binds to the InsP3R and increases its activation, leading to dysregulated calcium release, excitotoxicity, and striatal cell death (Tang et al., 2005; Tang et al., 2003). His lab continues to investigate the mechanisms linking InsP3R overactivation with HD pathology.

A recent focus is store-operated calcium (SOC) entry, a pathway in which a protein called STIM helps to regulate the amount of calcium entering synapses from the extracellular space. In collaboration with EnVivo Pharmaceuticals, they are testing blockers of SOC entry as a means to protect against synaptic dysregulation and cell death. Using screens similar to those described by Juan Botas, they found one compound, EVP4593, that rescued climbing behavior in the flies. EVP4593 also rescued increased calcium entry in the brains of YAC128 mice, protecting against dysregulation and death of striatal cells. Similar compounds were neuroprotective in cultured medium spiny neurons from YAC128 mice, via prevention of calcium entry.

An early observation in HD brains was the significant loss of dendritic spines (Graveland et al., 1985), which form important communicative points between neurons. To explore treatments to combat loss of spines, the Bezprozvanny lab has recently created a cellular model of spine loss in co-cultured cortical and striatal neurons from YAC128 mice. They have uncovered several interventions that block SOC influx and protect against spine loss, including

genetic knockdown of IP3 receptors with antisense oligos, or knockdown of STIM2 with RNAi. Furthermore, overactivating STIM2 in normal neurons leads to spine loss, further confirmation that excess SOC entry is detrimental. They can also rescue spine loss in YAC cells and in vivo by treating with EVP4593. STIM2 regulation of calcium signaling also arose in Juan Botas's work, as well as through a screen by EnVivo, suggesting that this pathway may be a good therapeutic focus.

Another project in the lab aims to understand the mechanism of pridopidine, which has recently entered clinical trials for HD. Though it was introduced as a dopamine stabilizer, it actually has a stronger affinity for the sigma-1 receptor (S1R), which also modulates calcium signaling. Bezprozvanny's group has found upregulated levels of S1R in HD mouse brain, contributing to spine loss. Pridopidine appears to prevent spine loss in MSNs from YAC128 mice, also inhibiting SOC entry. One possible mechanism involves S1R binding to STIM (Srivats et al., 2016). It is also possible that S1R inhibits InsP3R activation in medium spiny neurons. The lab is currently using targeted mutation techniques to determine exactly how pridopidine binds to the S1R. Overall, this work contributes to our understanding of calcium dysregulation in HD.

Concluding Remarks

Participants in the HD2016 meeting came together in a supportive and generative environment to share a diverse array of techniques and theories. From the historical roots of HD research to emerging data from clinical trials, our understanding of human genetics and unwavering support from the HD community has converged upon therapeutic approaches that were mere ideas only a decade ago. The HDF will continue to support innovative research that strives to overcome the great challenges of the field: analyzing vast datasets, identifying the best pathogenic targets, translating data from models to humans, and perfecting drug delivery to the brain. Participants were inspired to persist in their efforts alongside HD families and to forge institutional and international partnerships that will speed our search for a cure.

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