CHDI Workshop Report: Role of Mutant Huntingtin RNA in HD Pathogenesis
Nov 9-10, 2011, New York
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Executive Summary

Huntington's Disease (HD) arises from a CAG expansion, which encodes a poly-glutamine stretch in the gene (Htt) for the huntingtin protein. The protein remains enigmatic; it is ubiquitously expressed throughout the body over the entire lifespan, and it appears to play an increasing number of roles in cellular function, perhaps including intercellular trafficking and possibly gene regulation. One question that remains outstanding is whether the deleterious effects of HD arise from toxic gain- or loss-of-functions at the protein level, or whether mutant Htt RNA itself confers toxicity. This and other questions surrounding mHtt RNA were the focus of this Workshop.

RAN Translation

Laura Ranum described her experiments that led to the discovery of RAN translation, or repeat-associated, non-ATG-dependent translation. Her lab has been working on a gene they identified in 1999 that causes spinocerebellar ataxia called SCA8. The mutation is a CTG/CAG repeat expansion. Initially, it was somewhat controversial whether this expansion mutation was truly a disease-causing mutation, because people with this mutation often have reduced penetrance, in other words many people with SCA8 mutations don't get sick. To address these controversies and to learn more about the disease, Ranum's group made a mouse model of SCA8 using a large insert bacterial artificial chromosome (BAC). The transgenic mice expressing the SCA8 mutation got sick: they developed motor coordination deficits and aberrant cerebellar electrophysiology. While it was initially recognized that CUG expansion transcripts were expressed in these animals, further characterization of these mice and human tissues led to the discovery that expression also occurs across the expansion mutation in the CAG direction and that the CAG expansion transcripts encode a nearly pure polyglutamine protein which accumulates in Purkinje cells. The gene expressed in the polyglutamine encoding CAG direction is called ATXN8 and the gene expressed across the repeat in the CUG direction is called ATXN8-OS.

This was the first report of a repeat expansion mutation in which transcripts spanning the mutation were shown to be expressed in both directions. In a paper published in PLoS Genetics, Ranum showed that CUG transcripts form RNA foci in Purkinje cell nuclei, and described several lines of evidence that RNA gain of function effects contribute to the

disease. Ranum's hypothesis from this work was that spinocerebellar ataxia 8 (SCA8) involves both RNA- and protein-mediated gain of function effects.

To understand the role of the ATXN8 polyGln protein in SCA8, Ranum and colleagues mutated the only ATG initiation codon 5' of the CAG expansion. Surprisingly, Western blots and 1C2-antibody staining showed that the protein was still produced. Through a large number of additional experiments, the group showed that this non-ATG translation can occur in all three reading frames across both CAG (polyGln, polyAla and polySer) and CUG (polyLeu, polyAla, polyCys) in transfected cells that this process is favored by long, hairpin-forming repeat tracts. Mass spectroscopy identified a series of polyAla proteins and suggested that translation in the polyAla frame initiates at sites throughout the repeat sequence itself. Many questions still surround this newly described Repeat-Associated Non-ATG-dependent (RAN) translation, not least of which is how this novel type of translational initiation occurs, as it appears to substantially differ from the well established Met-initiated translation.

Other questions arise as well. What are the implications for disease, and for HD in particular? If other novel proteins are being made in humans in these diseases, researchers should be looking for them, Ranum said; this was a major point of her paper. In order to determine whether RAN translation could occur in disease, Ranum created antibodies that detect the C-termini of two predicted RAN-translated proteins: a novel SCA8 polyAla expansion protein and a novel DM1 polyGln protein. After many experiments, they have shown by immunofluorescence that the SCA8 polyAla accumulates in Purkinje cells in mice and humans and that DM1 polyGln is detected in DM1 mice and patient tissues.

Tobin pointed out that these revelations have significant implications even simply for animal models of HD. For example, the BAC-HD mice are made with a long sequence of human DNA, but it uses a mix of poly CAA and CAG codons, whereas the YAC is made with the human pure-CAG sequence. Interestingly, the YAC seems to have an earlier disease onset and other phenotypic distinctions. The models have many differences, but the finding that poly-CAG repeats present a site for "promiscuous initiation," the sequence needs to be considered. Ranum has examined translation differences with pure CAG, pure CAA, and is currently working on various mixes of CAG and CAA; she will report on those findings to CHDI. Another interesting characteristic of the RAN translation is that it appears not to occur in every type of cell. She has shown that RAN translation can occur and that protein accumulates in Purkinje cells. In myotonic dystrophy tissue, they made another antibody to detect the presence of putative polyglutamine protein. They found evidence for polyglutamine protein expressed only at relatively low levels in skeletal and cardiac muscle, but higher levels in blood.

Additional studies are needed to determine what role this novel polyGln expansion protein may play in DM1. Ranum and Nancy Bonini discussed how the unorthodox translation might occur. Rather than being triggered by the mRNA's sequence, the hairpin structure that arises from the repeats might be what the ribosome is recognizing and responding to. Perhaps the ribosome simply stalls out at that structural feature, and that triggers translation. The ribosome uses a "scanning" mechanism, and the initiation factors detect structural areas of high or low energy. The dependence of the RAN translation on CAG-repeat length seems to support this view. With only 15 or 20 CAG repeats, the

polyglutamine (and other residues) are not produced. With increasing repeat length, glutamine protein products can be detected, and Ranum has evidence in cell culture that, as the repeat lengthens, higher levels of proteins translated from all three frames accumulate. She suggests that this could in theory underlie the varying degrees of disease severity with increasing repeat lengths.

Kevin Weeks asked how efficient the RAN translation process looks relative to "standard" translation; Ranum reported that it can operate as efficiently as translation initiated at the AUG site in at least some constructs, but this is an important question that needs further investigation. Weeks asked whether RAN translation might possibly work like an internal ribosomal entry site (IRES), similar to those found in viral RNA? Possibly, Ranum said, considering the diversity of described IRES mechanisms. Melissa Moore also described a translation process found in *E. coli*. Although many of these alternate translation mechanisms have not been found to occur in mammalian cells, they should not be ruled out because the translation machinery is so highly conserved. Weeks went on to point out that "there's good evidence that all [these mechanisms] are putting some mRNA element in the cleft to get ribosome translation started." Finally, Ranum suggested that most of genome could be bi-directionally expressed, and scientists don't yet fully appreciate the complexity of gene expression. In fact even our definitions of what qualifies as a gene and how many genes are contained in the genome have become quite fluid. Alternative splicing, IRES mechanisms, and RAN translation all contribute to additional layers of complexity.

Allan Tobin pointed out that in the morning we had two potential start sites for Htt translation—the canonical AUG initiation complex and an upstream open reading frame (ORF)—and we now potentially have three or more additional start sites, if RAN translation occurs on Htt. If one envisioned a single molecule of Htt RNA associated with a ribosome, could the multiple initiation mechanisms be used simultaneously on that mRNA molecule?

Melissa Moore and Laura Ranum outlined the possibilities. One well-established mechanism of translation initiation involves the binding of a number of initiation factors to the 5' 7mG cap on the mRNA, the recruitment of the small ribosomal subunit, and a scanning process along the RNA until it encounters an AUG. Ranum described that interaction as "possessive;" extraneous translation events would not likely occur on that mRNA during canonical translation. However, the RAN translation or other IRES-like processes would likely not require ribosomal scanning; it works as a different kind of process. The on- and particularly off-rates of mRNA-ribosomal associations likely determine translation events. Moore postulated that "something about the RNA structure, and possibly that of the proteins it's bound to, creates a 'Velcro' for the ribosome." If it can bind and stay bound long enough, kinetic competition will decide whether translation is initiated.

As for the upstream ORF, competition also determines the outcome. At the stop codon, the RNA either stops and leaves the ribosome, or it goes on translating. In general, she explained, upstream ORFs tend to decrease translation of the downstream protein, because they initiate nonsense sequences. It's of note that the upstream ORF exists in Htt, because it indicates that the cell cares keeping Htt protein production in check, she reasoned. "If a cell wants a lot of a protein, it does not put an upstream ORF in front of it." Ranum reported that she did not yet know whether mutant Htt translation is primarily initiated at the upstream ORF or at the AUG site. And it has yet to be determined whether RAN translation occurs on

either Htt or mHtt. The answers might have important implications. Ethan Signer wondered in particular whether significant levels of cellular huntingtin might be "running around without the [amino acid] 1-17 frame," which would have extremely important functional consequences for the protein and probably the cellular biology.

RNA Toxicity

Wlod Krzyzosiak described his structural biology approach to understanding triplet repeat diseases. From the early 1990's, the diseases were known to arise from expanded repeats of the triplet sequence CAG, CUG, or CGG. In all three cases the RNA structure forms hairpins. If CUG and CGG repeats contained in non-translated RNA sequences are sufficient to cause disease, why should the translated CAG repeats be toxic only at the protein level? Perhaps the RNA might also contribute to the disease process. Based on structural analysis, the RNA hairpins seemed to differ in length and in the details of the hairpin stem architecture but not in their overall shape.

Might the repeat-laden RNA molecules recognize the same RNA-binding proteins in a common way? To address this question, Krzyzosiak selected the most frequent repeat alleles in normal population and patient-derived mutant alleles, transcribed DNA to RNA, and studied the structures of messenger RNA repeat regions. All the repeats formed a central hairpin, and in some cases flanking sequences contributed to this structure. In the case of the huntingtin transcript, an additional, shorter, and less polymorphic CCG repeat is separated from the canonical CAG repeat by 12 nucleotides of a specific sequence. It appears that the Htt repeat hairpin is composed of three sections. In the bottom section, both CAG and CCG repeats interact with each other. In the central section, CAG repeats are partially base-paired with the specific sequence, whereas the upper part of the HTT hairpin is formed by the interacting CAG repeats only. Normal Htt transcript differs from the mutant transcript only in the length of the upper part of the hairpin, and its regional stability is affected by CAG repeat length. This information was derived from chemical and biochemical structure-probing and structure-melting experiments.

More recently, Krzyzosiak has investigated the effects of CAG repeat-laden RNA on alternative splicing of genes that undergo aberrant alternative splicing in myotonic dystrophy type 1 (DM1) cells expressing expanded CUG repeats. He wanted to find out whether the alternative splicing events might contribute to pathogenesis in polyglutamine diseases. Experiments in patient-derived fibroblasts and HeLa cells expressing exogenous repeats suggest that expanded CAG repeats trigger aberrant alternative splicing of several tested genes including insulin receptor (INSR) as well as chloride channel 1 (CLCN1) and sarcoplasmic/endoplasmic calcium ATP-ase 1 (SERCA1). The splicing factor responsible for those events is muscleblind protein 1 (MBNL1). Krzyzosiak showed that mutant Htt transcript co-localized with MBNL1 and formed foci in nucleus, perhaps sequestering it and rendering it incapable of participating in RNA splicing. The appearance of nuclear foci draws a clear mechanistic similarity between CAG repeat-expansion diseases and DM1. The CAG repeats and CUG repeats of the same length differ only slightly in their MBNL1 binding ability. But in patient cells, mutant CAG repeats are generally shorter than mutant CUG repeats, and therefore display lower MBNL1 sequestration capacity. Another important point is that Htt RNA must be transported to the cytosol for translation, so the retention of RNA in the nucleus where the missplicing occurs is likely only temporary. Therefore, the alternative splicing aberrations caused by mutant CAG repeats may not have the same

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impact as those triggered by mutant CUG repeats. Photo-bleaching experiments might reveal the dynamic movement of RNA and MBNL molecules.

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Newly developed tools such as the GFP-like RNA sequence molecule called Spinach might also reveal information about these interactions, although it would be preferable to study endogenous RNA rather than the transgene required by Spinach. MBNL in fact is thought to shuttle the Htt transcript from nucleus out to its cytoplasmic site of translation. From Nancy Bonini's work, MBNL appears to have multiple types of effects in flies, likely in various cellular locations. Possible RNA-binding protein partners of Htt should not be limited to MBNL, which itself has three isoforms (MBNL1, 2 and 3). Other proteins to be considered include Fus and TDP43. Ramee Lee pointed out that because mHtt RNA contains CAG that tags it for nuclear export, HD RNA toxicity might look quite different from myotonic dystrophy. By looking only for mis-splicing readouts, we might be missing other distinct forms of RNA toxicity in HD. What could the RNA foci distribution and composition in HD cells tell us about RNA toxicity? Experiments could now be done using HD patient tissue to look for missplicing events, associated with MBNL and other factors. Another factor to consider is that Htt RNA can encode a polyglutamine tract with a pure CAG repeat sequence or with CAGs interrupted by CAA, which would affect the structure of the RNA hairpin that forms. Ethan Signer added that knock-in mice expressing each type of transcript have been prepared and are now being analyzed.

RNA-Binding Proteins

Clotilde Lagier-Tourenne (from the lab of Don Cleveland) spoke about her work investigating the RNA-binding proteins TDP43 and Fus in amyotrophic lateral sclerosis (ALS). TDP43 is found mainly in the nucleus, but in most cases of human ALS, TDP-43 has been found aggregated in the cytoplasm. Dominant TDP-43 mutations have been identified in only about 5% of familial cases of ALS, and 1% of sporadic patients, but the mislocalization is seen in all sporadic cases. In experiments using RNA-seq, junction arrays, and CLIP technology, Lagier-Tourenne and colleagues sought to determine what sort of RNA species TDP-43—and now Fus—were interacting with that might hint at their potentially multiple, diverse roles. She found that TDP-43 binds thousands of targets, mainly in noncoding regions of RNA with very long introns.

The Cleveland lab has used antisense oligos to knock down TDP-43 by 80% in mouse brain, delivered by stereotactic injection in the striatum. The aim was to correlate TDP-43 binding sites to the changes in RNA species after knock down. Some TDP binding sites may mediate splicing events, but others were far away from an exon-intron junction, and might serve another purpose. Roughly 300 genes were upregulated in the absence of TDP-43, while another 200 were downregulated, and these differed in binding strength to TDP-43. Those RNA species that were reduced after TDP-43 knockdown bind the protein at multiple sites, whereas the upregulated genes form weak interactions with TDP-43. Although the binding patterns differ for Fus, this similar pattern of downregulation of highly bound partners seems to be emerging. Fus also binds RNA of long-intron genes, and shows a good deal of overlap with genes bound by TDP-43. Melissa Moore added that the emerging data around TDP-43 indicates that the protein needs to be maintained in a narrow concentration range. Mouse models now show that either over- or under-expression of this ubiquitous protein is toxic and leads to neurodegeneration.

Lagier-Tourenne then discussed the recent breakthrough in the genetics of ALS with the discovery of an abnormal repeat expansion on chromosome 9 as the most common cause of familial ALS. Further studies will be necessary to determine how this GGGGCC expansion in an intronic region of the gene C9orf72 triggers disease. However, the nature of the mutation suggests it echoes a known mechanism of RNA toxicity: a loss of function arises from RNA-binding proteins being trapped by the expansion.

RNA Structure

Kevin Weeks described his work developing a "long-term vision for thinking about RNA as a therapeutic target," using what he calls SHAPE technology. (SHAPE stands for selective 2' hydroxyl acylation analyzed by primer extension.) ts is agnostic about whether repeat-associated disease toxicity is RNA-mediated, protein-mediated, or both. But if RNA might be used as a target, it needs to be in an allele-specific way. The work he described is distinct from other methods that target SNPs associated with long or short alleles, which he calls a promising but necessarily limited strategy. Instead, Weeks is aiming to rapidly generate high-quality information about the global RNA fold of Huntington-related RNAs. The SHAPE technique exploits the ability of simple reagents to react broadly with RNA the 2'-hydroxyl group. Therefore, by looking at this position with acylation and primer extension, it is possible to "see" all the nucleotides of an RNA species at once. They have focused on huntingtin (Htt), starting with what Weeks called "the naïve experiment to look at synthetic transcripts *in vitro*," eventually the goal is to look at RNA in cells.

RNA Quality Control

Melissa Moore discussed her work investigating RNA quality-control pathways. In particular, Moore's interest lies in a group of diverse, specialized processes called mRNA translation-dependent decay (TDD), which she now prefers to call translation-activated self-destruction (TASeD). The first, called nonsense-mediated decay, (NMD), occurs when an mRNA stop codon appears in an inefficient context for termination. In a second process called non-stop decay (NSD), the mRNA has no stop codon, so the ribosome stalls at the end of the mRNA and needs a special mechanism to get the ribosome off the mRNA, which then gets enzymatically decayed. Here she focused on a third process called no-go decay (NGD), discovered by Roy Parker.

Ribosomes have three sites termed A, P, and E. Transfer RNA (tRNA) shuttles a new amino acid into the ribosome's A site; the P site contains a tRNA with its growing peptide; and tRNA exits the ribosome at the E site. In the case of NMD, the A site of the ribosome contains an inefficient stop codon; in NSD it contains no codon. Parker asked what would happen if translation stalled out in the middle of an ORF at a normal, non-stop codon? Using a yeast construct, he inserted a very long GC base-paired stem to create an obstacle that would stall the ribosome's progress and found that the mRNA was cleaved upstream of the impeding structure and was targeted for decay. Moore then wondered, what if the impediment occurred as a function of ribosomal, rather than mRNA, structure? Would the cell differentiate between the two, or would it "throw the baby out with the bathwater," and degrade the whole complex? She made mutations in the ribosome's small subunit that prevented the normal conformational change that occurs when a correctly base-paired tRNA enters—thereby creating a ribosomal deficiency so that it can initiate but not elongate. The small subunit was then degraded in a process Moore called non-functional rRNA decay

(NRD). Various interacting factors are required for each of these RNA-degradation pathways. NMD uses factors called Upf1, 2, and 3, which interact with release factors. For NSD, a protein called Ski7 looks like a release factor, binds to the empty A site, and recruits an exosome to degrade the RNA. NGD and NRD require the proteins Dom34 and Hbs1, which bind in the A site of the stalled ribosome and act as release factors.

How do these translation-dependent RNA degradation processes relate to HD? Neil Aronin came to Moore with his observation that he could detect more mutant Htt RNA in cells than normal-length Htt RNA. How might the decay kinetics differ between the two forms of Htt RNA? Moore suggested that the long repeats might cause the ribosome to stall out, either by forming an interfering structure or simply by slowing elongation at the repeat stretch. In a 2005 *Gene Expression* paper, Peter Detloff showed that when cells expressing constructs with long CAG repeats were deprived of glutamine, the mRNA containing the long repeats were decayed preferentially over other mRNAs. In yet another decay process, the cell responds to inefficient codons.

A recent paper in yeast showed that when a ribosome comes to a stretch encoding a particular amino acid—in this case arginine—the codon-specific tRNA is the limiting factor, and the ribosome has to slow down according to tRNA availability. Various protein complexes, some of which mimic tRNA, compete for access to the ribosome's A site, so if translation slows down, the probability of mRNA cleavage and degradation increases. These factors include the yeast proteins Dom34 and Hbs1; manipulation of these factors provides a way to probe the decay mechanisms at play. To address the question of the decay kinetics of mutant vs. wildtype Htt RNA, Moore has undertaken polysome profile studies in R6/2 mouse brain tissue. To create a polysome profile, before lysing the cells one would normally treat with cycloheximide to "lock the ribosome onto RNA," Moore explained. "But if the ribosome is already stuck, you don't need to fix with cycloheximide first." Moore and Aronin wanted to determine whether the mRNA with expanded CAG remains associated with the ribosomes, whereas those without expansion don't. Other data from Jenny Morton and Tony Reiner suggests that mHtt with CAG repeats above 200 are less toxic; the medium-length repeats appear to be most toxic. Multiple possibilities could explain this finding: the RNA is no longer transcribed, or perhaps the RNA is subjected to no-go decay. Moore suggested that screening for compounds that increase NGD, perhaps even slightly, could be helpful for preferentially eliminating mutant Htt mRNA. Cell-based assays could screen out toxic compounds. This approach could be combined with Matt Disney's approach of stabilizing the RNA structure with small molecules; stabilization of the repeat's hairpin structure might stall the ribosome and promote NGD. Key questions must first be addressed. First, is mHtt subject to NGD, and second, is mHtt decay kinetically faster than that of wild-type? Finally, can we shift that rate with various factors?

Huntington's Disease-like 2 (HDL2)

Dobrila Rudnicki discussed her work with Russell Margolis in Huntington's Disease-like 2 (HDL2) in the context of the workshop's revelations. HDL2 arises from a CTG/CAG expansion mutation in the alternatively spliced gene junctophilin 3 (*JPH3*). *JPH3* encodes a protein that's hypothesized to link the endoplasmic reticulum to the plasma membrane and regulates neuronal calcium flux. A full-length version of *JPH3* does not contain the repeat region. There are at least three *JPH3* splice variants; the repeat can appear in the 3' UTR region, or in frames that encode poly-alanine or poly-leucine. Similar to HD, a prominent

feature of HDL2 neurons is 1C2 antibody-positive inclusions, initially leading to a hypothesis that HDL2 is a poly-glutamine disease. While an existing mouse model shows that it's possible to get polyglutamine expression from the strand antisense to *JPH3*, there is so far no evidence that expanded polyglutamine is indeed expressed in HDL2 brains.

However, in addition to protein inclusions, HDL2 brains were found to contain RNA foci detectable with *in situ* riboprobes specific to expanded CUG repeats. This suggested that RNA neurotoxicity might contribute to HDL2 pathogenesis. Ethan Signer wondered whether the huntingtin protein plays a role in HDL2 pathogenesis; this has not been definitively answered but could be addressed, among other approaches, by using interfering RNA (RNAi) directed at Htt. Rudnicki and Margolis proposed three possible, non-exclusive mechanisms for HDL2 pathogenesis, each of which is supported by some evidence: RNA neurotoxicity; loss of function of the full length JPH3; and gain-of-function due to expression of expanded polyglutamine. HDL2 presents a unique opportunity for further exploration of HD pathogenesis, as searching for converging pathways of pathogenesis may help us better understand both diseases.

Round-Table Concluding Discussion

Russ Margolis plans to develop the possibility of using the endogenous antisense transcript at the HD locus to target Htt. "We have number of things to flesh out," he said, and he now plans to go in some different directions based on what he's learned here. One approach is to develop a simple assay to search for factors that drive the antisense promoter. The question is whether it is worthwhile to perform a high throughput screen for small molecules that drive the promoter and hence increase expression of the antisense gene. Opinions vary about the answer. As for HDL2, he sees much work to be done and wants to track in parallel with what's being done in HD. Useful endeavors might be using transcriptome and proteome analysis of both diseases to try to come up with better models and to do better comparisons of HD and HDL2. Junctifilin3 is an obvious target for comparison.

Nancy Bonini finds many potential synergies here, which the discussion has helped catalyze. "One never knows where a cure's going to come from," so one has to do the targeted approaches but also develop "wildcard approaches." In terms of specific experiments, she would like to explore different therapeutic approaches to targeting RNA structure, because she has a lot of information about RNA structure in the fly linked to specific phenotypes. She is interested in the range of potential toxicities of RNA, and will continue to investigate those.

Neil Aronin believes the "wildcard" for HD therapeutics could be Matt Disney's potential small molecules targeted at RNA. "They make sense," in terms of targeting both sequence and structure. Aronin hasn't been dissuaded from his current research approach (of examining mutant and wildtype Htt allele transcripts and their relative quantities and stabilities), but Aronin will take his research in new directions based on what he has learned here. The RAN (repeat-associated non-ATG) translation is intriguing, but we need to make sure it applies to HD, at least in a cell model with an appropriate readout of toxicity. It's not enough just to show a binding interaction, or even that another transcript (of Htt) is translated. To assess any pathological role, it has must be assessed in a biological system.

Wlodzimierz Krzyzosiak focuses on microRNAs, both natural and artificial. Natural miRNAs can regulate Htt transcript. After predicting how these microRNAs might regulate Htt at various sites, he is now in the process of doing experiments to test them. With regard to the artificial microRNAs, those can be targeted to repeats regions for therapeutic purposes. After identifying interactions, the best candidates could be put into vectors for long-lasting expression in cells and in animals. Krzyzosiak also continues to work to understand HD pathogenesis: what is inside those RNA foci? And what are the remaining unidentified mis-splicing events that occur with Htt?

Chris Ross was already thinking about doing RNA seq experiments, and now has a better appreciation of the complexities associated with that technology. Perhaps a better first approach would be to use exon-junction arrays. Another important descriptive question—what's in the RNA foci?—could and should be addressed by biochemical and analytic methods. Ross wants to continue to study the role of RNA metabolism in HD toxicity. Partly based on Naoko Tanese's comments, Ross believes it's important to study RNA message as well as protein within granules. In addition to molecules they have examined, he wants to examine Fus and TDP43 for a potential role in toxicity. An experiment to see the effects of having both CAA and CAG within the repeat is another obvious, important one. Finally, finding ways to manipulate the protein separate from the RNA could answer fundamental questions about RNA toxicity and metabolism in HD—perhaps by manipulating the lysosome. Ross is also excited about an RNA-targeted small-molecule approach. If that could alter toxicity in HD cell models, "we could really move [therapeutics] from RNA structure to function."

Dobrila Rudnicki found the RNA-focused meeting very useful. She's excited to see people interested in HDL2. She'll continue with the projects she's been working on to find RNA-binding proteins. As for lowering mutant Htt using antisense oligonucleotides, Rudnicki believes that it is at least useful to model HD toxicity.

Clotilde Lagier-Tourenne believes that we need more direct evidence to show RNA toxicity in HD. We shouldn't assume that what's the case for muscular dystrophy will hold true for HD, or even that the same RNA-binding proteins are involved. Another point to remember: mice express very long CAG repeats, so we might see things—like foci—in animals that we don't see in HD patients with only a 40 CAG-repeat expansion. Finally, Lagier-Tourenne emphasized that the normal function of the Htt protein is still not known with respect to RNA processing. A better understanding of wildtype huntingtin would help to determine any potential disturbance of RNA processing by the polyQ region, either at the protein or the RNA level.

Matt Disney is willing to contribute to efforts to develop small molecules that might target any mechanism associated with the CAG repeat. He was intrigued by the idea of NGO decay, perhaps stimulating or allowing cleavage of mutant Htt to a higher degree than the wildtype RNA. "Perhaps a small molecule could stimulate a bigger barrier for NGO decay." He was also intrigued by the idea of a theoretical ensemble of different RNA structures (described by Kevin Weeks). He has not yet explored the possibility of a small molecule that might stop translation. Disney also believes CHDI should invest in Moore's nonbiased screens. Such experiments might reveal other mechanisms by which a small molecule could stop production of mutant but not wildtype Htt. "Places like Scripps are well positioned to do

that," he said. An additional potential benefit from unbiased screens is that you may come up with interesting, unanticipated results.

Melissa Moore agrees that for therapeutic approaches, we have to pursue multiple approaches. She advocates for continuing "full guns" with mutant-allele-specific Htt silencing either by si or shRNA technology. Antisense oligos directed at the CAG repeat also still seem worth pursuing. Moore is particularly excited about Disney's small molecules, and has discussed with him other ways to get the molecules to perhaps self-assemble within cells, or attract nucleases. Additionally, if there were any way to selectively inhibit transcription of the expanded CAG allele, that seems promising but she doesn't know how that could be done. The avenue of up-regulating endogenous RNA quality-control mechanisms, such as NGO decay, could be explored with some simple experiments: up- and down-regulate NGO decay in cells to see if it affects Htt expression levels. The question of RAN translation will also be critical to answer in HD: it may be that cells are also making other protein species that could be just as toxic as polyQ itself. She feels we also need to determine whether cells are making truncated Htt that are missing the first 17 AAs. Finally, other neurodegenerative diseases have benefitted recently from using new yeast models as fast screens for cell toxicity in particular. This sort of system is distinct from yeast-two-hybrid systems to find protein interactions or other older models. Although most yeast systems use only exon 1 from Htt and don't have pure CAG repeats, it might provide a good screening tool. Moore suggests contacting Aaron Gitler at the University of Pennsylvania.

CHDI scientists added their impressions from the meeting.

Ramee Lee noted that although CHDI is already targeting mRNA, there's still so much to learn about the basic biology of RNA, from the newly described RAN translation to ribosomal stalling and endogenous degradation mechanisms. She could see many potential therapeutic targets arising from these research lines. Armed with new tools and information she believes we can synergize to accomplish a lot in the next year to two.

Ethan Signer was struck by two particular avenues. One, if NGO RNA decay could be boosted—even a little bit—that could have big effects in a cell. Second, the RAN translation is intriguing, but he views it from a slightly different perspective than looking at the downstream results of distinct protein species. If RAN translation occurs on Htt, that signifies that there is an inherent recognition site. "Something makes the translation machinery respond to certain loci. Irrespective of what happens after they're recognized, what makes them be recognized?" Whether the hairpin structure or some other element comprises the recognition site, perhaps we could harness that repeat-specific site and use it to target therapies.

Bernhard Landwehrmeyer was impressed by the enormous knowledge base about RNA structure, which he didn't appreciate and opens many new potential therapeutic strategies. Kevin Weeks's approach of generating small molecules to interfere with the various structural species he found particularly intriguing. He would like to explore the important connection back to HD patients: could we find evidence for these disease mechanisms in biopsy or postmortem tissues taken directly from patients? For example, perhaps we could identify RNA foci within tissues. And then, regardless of whether we can establish that RNA toxicity *per se* plays a role in HD pathology, he would like to underline the importance of

intervening in HD very early, where both RNA- and protein-mediated toxicity might have effects. “If toxicity isn’t a part of the disease, there’s still no reason that we shouldn’t figure out how to intervene by targeting RNA, and also figure out what it does do in the processing of Htt.”

Allan Tobin found a couple of important lessons for CHDI. He now realizes that the new technologies, ideas, and discoveries discussed here not only allow exploration of new areas, they also provide a better understanding of things we thought we understood, and even based big programs on, that we may not have understand at all. The RAN translation idea and the idea of small molecules that could interfere at the RNA level are both new and important ideas for CHDI. This workshop has illustrated the importance of keeping up the Early Discovery Initiative work, because there are always discoveries that change our view of biology and of HD.