Québec: Teasing Out the Function of TDP-43
Part 1 of a 3-part series.

4 October 2010. Sponsored by the Fondation André-Delambre and now in its sixth year, the Symposium on amyotrophic lateral sclerosis is rapidly becoming one of the top tickets in ALS research. The meeting, held September 24-25 at Université Laval in Québec City, Canada, attracted 80 scientists who discussed cutting-edge data on the mechanisms of the disease and progress on treatments and in the stem cell field. "Speakers presented an avalanche of results that we have to think about," said **Jean-Pierre Julien** of Université Laval in a closing statement. Julien co-organized the meeting with **Jasna Kriz**, also at Laval.

A theme that stood out was TAR DNA Binding Protein 43 (TDP-43). Its role in amyotrophic lateral sclerosis (ALS) is well-accepted—but what does TDP-43 actually do? For some time, the answer has been a vague, "something having to do with RNA." In Québec, researchers presented a couple of approaches that may yield more concrete answers.

Clotilde Lagier-Tourenne of the University of California in San Diego asked which RNAs TDP-43 interacts with. Lagier-Tourenne, who works with Don Cleveland, is collaborating with Maddalini Polymenidou from the same group and the laboratory of Gene Yeo, also at UCSD. The researchers are using a relatively new technique called cross-linking immunoprecipitation and sequencing (CLIP-Seq) to identify such mRNAs in mouse brain. They treated the mouse brain samples with crosslinkers to cement any otherwise fleeting RNA-protein interactions. Then, they used TDP-43 antibodies to pull down the protein and any associated RNAs. Finally, they sequenced the nucleic acid to determine which RNAs bind TDP-43. Further, they determined specific nucleotide sequences that recruit TDP-43. Scientists suspect the protein is involved in mRNA splicing, so it was no surprise to Lagier-Tourenne to find that these binding sites are in introns and in close proximity to intron/exon splice junctions.

One particular topic of debate in this field is whether ALS pathology is related to TDP-43's normal role. To begin to tackle this question, Lagier-Tourenne and Polymenidou, in collaboration with ISIS Pharmaceuticals Inc. in Carlsbad, California, used antisense oligonucleotides to knock down TDP-43 expression in mouse brains. By sequencing cDNA, she found changes in mRNA expression and splicing in these animals. The researchers are working to identify genes influenced by TDP-43 in the hopes of understanding how TDP-43 mutations cause ALS.

While Lagier-Tourenne is puzzling out TDP-43's role at the molecular level, other scientists are examining its effects in the whole organism. ALS researchers are steadily accumulating a fine collection of TDP-43 model mice (for a recent summary, see ARF ICAD conference story) and the meeting brought further updates from both the Cleveland and Julien labs.

Many researchers have expressed human TDP-43 in mice under the powerful, nervous system-specific PrP or Thy1 promoters. Julien, along with his colleague Vivek Swarup, took a different tack by cloning the entire human gene, native promoter included, and inserting that into their mice. In addition to making mice with wild-type human TDP-43, the researchers used site-directed mutagenesis to create the disease-linked A315T and G348C mutations in the transgene, creating additional TDP-43 mutant lines. The transgenic animals have low human TDP-43 expression throughout the body, Julien reported.

Both the wild-and mutant animals "look pretty normal," Julien said. They suffer neither paralysis nor early death, and their motor neurons remain undamaged up until 10 months, middle age for mice. They are wobbly when balancing on a rotarod, suggesting some loss of motor control. They also have memory problems that the researchers said reminded them of frontotemporal dementia, another TDP-43 proteinopathy. The mutants—particularly the G348C mouse—evince cytoplasmic aggregation and ubiquitination of TDP-43, similar to human ALS pathology. "One of the most striking changes in the mice is neuroinflammation," Julien added. The animals exhibit microgliosis and astrogliosis, providing further evidence that ALS has an immune system component.

Sandrine Da Cruz presented the Cleveland lab's latest and greatest on TDP-43 animals, covered last year on Alzforum (see ARF news story). In addition, the lab has been making animal models overexpressing fused in sarcoma (FUS), another gene linked to ALS. In all, the Cleveland group is juggling 36 mouse lines —18 each for TDP-43 and FUS transgenes including both wild-type and mutant versions (TDP-43 Q331K and M337V and FUS R521C and R514G). "The good news is there are a lot of mice," Cleveland said, adding in jest, "The bad news is, there are a really a lot of mice!"

The group took a two-pronged transgenic strategy for each gene. In some lines, the researchers expressed the human genomic version of FUS or TDP-43. In other animals, they used the PrP promoter and flanked the cassette with Lox sites, so they can selectively excise TDP-43 from different cells types using Cre recombinase. TDP-43 transgenic mice displayed gait abnormalities and muscle hyperactivity; FUS mice exhibited similar walking problems as well as astrogliosis and altered axon diameter.

Of all these mice, unfortunately none are quite the ideal model scientists are looking for. Echoing a long-standing difficulty in Alzheimer disease research, each strain models an aspect, or aspects, of the whole picture. In an email to ARF Julien commented: "The various talks on transgenic mice illustrated the difficulty in creating models that mimic the human ALS disease well." —Amber Dance.

Québec: Motor Neurons from Stem Cells—Really, Truly?
Part 2 of a 3-part series.

5 October 2010. From fibroblasts, to stem cells, to neurons—it is a vision that holds the promise of someday treating amyotrophic lateral sclerosis (ALS), or at the least helping

scientists to better understand the disease. At the Fondation André-Delambre's annual symposium, held September 24-25 in Québec City, Canada, several researchers reported progress toward that still-distant goal. Researchers are using embryonic stem cells as well as induced pluripotent stem (iPS) cells, (http://www.alzforum.org/new/detail.asp?id=2558"> see Alzforum series) to make motor neurons, which they then study in culture. Some are using these artificially made motor neurons to examine the key question of which cell types truly cause ALS. Others are applying stem cell tools to understand why motor neurons in particular are susceptible to degeneration. Another question is which kinds of motor neurons are in greatest danger. **Victor Rafuse** of Dalhousie University in Halifax, Canada, presented data on susceptible motor neurons that fatigue quickly versus resistant motor neurons that fatigue slowly. His results to date suggest, to scientists' dismay, that when they manufacture motor neurons from stem cells, the resulting neurons are most similar to the natural fatigue-resistant motor neurons—the ones least likely to degenerate in ALS.

Among the top news: **Kevin Eggan** of Harvard University announced that his long-awaited patient-specific iPS cell lines are available for the asking. He has both SOD1 and TDP-43 mutant varieties. "Much exciting research using this new model is expected in the future," wrote meeting attendee Christine Vande Velde of the Université de Montréal in Canada, in an e-mail to ARF. [For a comprehensive summary on iPS cells in neurodegeneration, see Madolyn Bowman's series on Alzforum .]

A central question in ALS research has been which cells actually inflict the damage. Motor neurons are the ones that die, but studies in some model systems suggest that glia instigate the disease in a non-cell autonomous fashion (see ARF News story on Nagai et al., 2007 and Di Giorgio et al., 2007; and ARF News story on Clement et al., 2003). One treatment possibility, then, would be to replace endogenous, damaging microglia with new ones that do not carry mutations. **Nicholas Maragakis** of the Johns Hopkins School of Medicine in Baltimore, Maryland, is working on transplanting glial-restricted precursor cells (GRPs) into rodents, where they differentiate into astroglia. In Québec, Maragakis reported that he is having some trouble getting human GRPs to differentiate in the same way, and to spread beyond the injection site. He suggested that in the meantime, the transplantation of GRPs into animals of different genotypes could be useful in the lab to understand how different cell types interact in the disease.

Much of the work Maragakis discussed was performed by Angelo Lepore, who derived GRPs from fibroblasts of mice harboring an ALS-linked mutation in superoxide dismutase 1 (SOD1), and injected them into the brains of healthy rats. This is a 'mechanical' counterpart to chimeras other researchers have created genetically using the Cre/Lox system to selectively excise or express mutant SOD1 (mSOD1) gene in different cell types. The injection protocol has the advantage that it transplants restricted cell lineages locally, Maragakis said. The majority of GRPs develop into astrocytes. In addition, he noted that scientists can do "mix-and-match biology," putting wild-type cells into one brain hemisphere and mutant ones into the other, for built-in controls. The disadvantage, he said, is that the injected cell type is immature and will not necessarily integrate with the native neural network.

So far, the researchers have observed the transplanted animals but for three months. In this time, they noticed ubiquinated inclusions, loss of motor neurons, and weakened grip in animals that receive mSOD1 GRPs. “This is not recapitulating ALS,” Maragakis said. “I think of it rather as influencing motor neuron vulnerability.”

For his part, Eggan presented preliminary work that may help scientists understand the relative contributions of motor neurons and astrocytes to ALS. Evangelos Kiskinis and Sophie DeBoer in the laboratory are working with long-term, time-lapse microscopy to examine how stem cell-derived motor neurons survive in the presence of wild-type or mSOD1 astrocytes. When the scientists plated motor neurons on wild-type mouse astrocytes, the neurons put out a dense network of processes over the course of two weeks. When plated on astrocytes from mSOD1 mice, the motor neurons initially behaved similarly. But after about a week, the neurons retracted their processes and died. “The cells never [achieve] the same complexity of processes that you see in the control experiment,” Eggan said. “It really does seem like we are [mimicking] some sort of effect—which is going on very early in the lives of these [ALS model mice]—in tissue culture.”

Yet another central question in ALS research is why the motor neurons, of all cells, are the ones that perish. **Christopher Henderson** of Columbia University in New York City is interested in how motor neurons respond to environmental toxins such as organophosphates. Used in solvents, plasticizers, lubricants, and fertilizers, organophosphates have come up as a possible explanation for high ALS rates among farm workers and Gulf War veterans (Horner et al., 2003). Using neurons derived from embryonic stem cells, he and colleague Marine Prissette found that motor neurons are more likely to die from organophosphate treatment than other types of neurons.

ES- or iPS-derived motor neurons have many advantages, including the ability to create patient-specific lines that carry human mutations associated with ALS (see ARF News story on Dimos et al., 2008), but it is not clear if cultured cells are really similar to the motor neurons that die in ALS. In fact, that might not be the case, Rafuse said. Motor neurons, he noted, are heterogeneous. Some fatigue quickly—such as the small motor neurons innervating the arm muscles we work at the gym—and others are fatigue-resistant—for example, those controlling the neck muscles that hold up our heads all day long. Fatigue-resistant motor neurons are less susceptible to ALS (see ARF News story on Saxena et al., 2009).

The standard trick researchers use to convert stem cells into motor neurons is to add the developmental regulators Sonic hedgehog and retinoic acid. Rafuse found that most motor neurons coming out of these protocols express the fatigue-resistant neuron marker **Lxh3**, not the fast-fatiguing marker Lim1 (Soundararajan et al., 2006). And the work of Sam Pfaff at the Salk Institute in La Jolla, California, suggests that when injected into animals, the stem cell-derived motor neurons grow toward fatigue-resistant postural muscles. What’s more, they even convert some fast-fatiguable muscle into fatigue-resistant muscle.

In other words, the stem cell-derived motor neurons scientists are studying may be those that are least susceptible to degeneration in ALS. “This work has important implications for the field, and we will need to keep this in mind as we re-interpret earlier works using these cultured neurons,” Vande Velde wrote.

“Excitingly,” Vande Velde added, “he has identified a ‘recipe’ to make large motor neurons, which are the ones lost in ALS.” Rafuse found that conditioned media from neural progenitors, in addition to Sonic hedgehog and retinoic acid, pushes stem cells toward the fast-fatiguing phenotype. Cells from these cultures express Lim1 and, when transplanted, grow toward fast-fatiguing muscles in the limbs. The work suggests that scientists will be able eventually to develop protocols for turning stem cells into motor neurons that are susceptible to ALS pathology.—Amber Dance.

Québec: In Zebrafish, Scientists See ‘ALS Matrix’
This concludes a 3-part series.

6 October 2010. In the race to understand the genetics of amyotrophic lateral sclerosis (ALS), the lowly zebrafish just might swim off with a share of the prize. At the Fondation André-Delambre’s annual meeting, held September 24-25 in Québec City, Canada, **Pierre Drapeau** of the Université de Montréal presented his work on genetic interactions between the top ALS-linked genes superoxide dismutase 1 (SOD1), TAR DNA Binding Protein 43 (TDP-43), and Fused in Sarcoma (FUS). By mixing knockdowns of those genes with overexpression of both mutant and wild-type versions in zebrafish embryos, Drapeau is building a data chart, or “matrix,” that shows which genes interact with each other. He concluded that TDP-43 and FUS work together in a pathway distinct from SOD1.

Why zebrafish? These finned vertebrates have much in common with humans. They share the same basic organ and tissue layout, and their proteins have 50-80 percent amino acid identify with those of people. Zebrafish TDP-43, for one, is 73 percent identical with the human protein. Conveniently for researchers, zebrafish embryos develop quickly; their first day is roughly equivalent to the first trimester for mammals. And when poked, they dart away, making it easy for researchers to see motor defects (Drapeau et al., 2002). They are also useful thanks to their simple neural physiology; each somite, or segment, possesses three motor neurons with long axons, again making it easy to spot motor neuron pathology. (Reviewed in Kabashi et al., 2010) and Best and Alderton, 2009.

Scientists have tools to knock down native fish genes or overexpress them, or even alter expression of multiple genes in the same embryo. By targeting ALS-linked genes in this fashion, Drapeau has created zebrafish that have motor and axon defects. “I am not saying this is ALS in the zebrafish,” Drapeau said, but “it recapitulates some features.”

However, there is a caveat to zebrafish studies, Drapeau conceded, whereby researchers may trade speed and convenience for direct disease relevance. “We have to keep in mind that we are dealing, potentially, with developmental defects, rather than

neurodegenerative changes," wrote Jean-Pierre Julien of Université Laval, who organized the symposium, in an e-mail to ARF.

In a recent study (Kabashi et al., 2010), Drapeau and colleagues examined TDP-43 function by overexpressing the human version of the protein. They used wild-type as well as three mutations that cause ALS in people: A315T, G348C, and A382T. They injected the knockdown or overexpression constructs into embryos one day post-fertilization, and analyzed them upon hatching a day later. The mutant genes caused short, overly branched motor neurons and hampered the fishes' swimming; overexpressing the wild-type human protein caused similar but milder pathology.

When the researchers knocked down the fish's normal TDP-43 expression, they observed the same axonal and swimming problems, indicating that both too much or too little TDP-43 is bad for embryonic development. Introducing human wild-type TDP-43, but not the mutants, into embryos was able to rescue the phenotype. The authors suggest that both loss of normal function and gain of toxic function may be at work in people with TDP-43 mutations.

In Québec, Drapeau reported on similar experiments using FUS genes. He used the wild-type as well as disease-associated mutations: R521C, R521H and a deletion after S57. Wild-type FUS was harmful only if expressed at high levels. The embryos were malformed and died. The mutant FUS transgenes impaired swimming at lower expression levels. R521H gave the most serious phenotype including shortened, over-branched axons, which was similar to that of the TDP-43-overexpressing fish.

When the scientists knocked down zebrafish FUS, they observed the same excessively branched axons and poor swimming. Over-expressing wild-type FUS rescued the knockdown embryos, but mutant FUS did not.

TDP-43 and FUS are both RNA-binding proteins (see ARF News story on Kwiatkowski et al., 2009 and Vance et al., 2009), and could conceivably be involved in similar processes. To analyze their relationship, Drapeau mixed the FUS and TDP-43 treatments together to start crafting his matrix. He found that human FUS rescued the motor neuron phenotype in TDP-43 knockdown embryos. However, human TDP-43 overexpression failed to rescue the FUS knockdown deficiencies. Drapeau concluded that the two function in the same processes. Since the presence of TDP-43 negates the need for FUS, FUS must be downstream of TDP-43. "They are working in one pathway," he said. "It does not matter where you hit it, you will interfere with it."

Genotype	+ WT hTDP-43	+ mutant hTDP-43	+ WT hFUS	+ mutant hFUS
TDP-43 knockdown	Rescue	No rescue	Rescue	
FUS knockdown	No rescue		Rescue	No rescue

Pierre Drapeau invited attendees to “enter the ALS matrix” with him as he detailed genetic interactions between TDP-43 and FUS.

In human ALS, TDP-43 pathology is common except in people who carry SOD1 mutations. When Drapeau studied SOD1 and TDP-43 or FUS mutations in the same animals, he found no genetic interaction between SOD1 and TDP-43 or between SOD1 and FUS. This confirms that SOD1 acts independently from the TDP-43 and FUS pathway, he said.

Next, Drapeau plans to use zebrafish with inducible TDP-43 expression to screen for drugs that rescue the phenotype (zebrafish drug screens reviewed in (Zon and Peterson, 2005). Since researchers have to check each fish for its swimming prowess, the process won’t be “high-throughput,” he joked. It’s more like “through-putt-putt.”—Amber Dance.