



Spinal Disease Protein

When normally folded, TDP-43 plays an important role in the body. Now, scientists are finding that when it is misfolded, the protein can wreak havoc.

By Karen Kreeger

Photographs by Addison Geary

If it is anything, science is incremental. It's a slow accumulation of knowledge punctuated by "eureka" moments. As the years go by, one of my favorite aspects of working in science communications is watching how discoveries unfold – and in the case of TDP-43, I mean that both literally and figuratively. TDP-43 is a normal protein that undergoes pathologic misfolding in its disease state.

As with all proteins, it is the shape of TDP-43 – the way the linear sequence of amino acids is ultimately folded into a three-dimensional protein – that is crucial in how it works or doesn't work. Normally folded, the TDP-43 protein is active mainly in the nucleus of cells throughout the body. It aids in editing the transcription of the genetic code.

A misfolded form of TDP-43 first became a major suspect in neurodegenerative diseases in late 2006, when Virginia

M.-Y. Lee, Ph.D., M.B.A., and John Q. Trojanowski, M.D., Ph.D., at Penn's Center for Neurodegenerative Disease Research and the Institute on Aging, made an important discovery. The husband-and-wife

team found mutated TDP-43 accumulated in post-mortem brain tissue from individuals who had been diagnosed with certain types of frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS, commonly known as Lou Gehrig's disease). The misfolded disease protein was recovered only from affected central nervous system regions, including the hippocampus, neocortex, and spinal cord. The approach that led to this discovery was an exploratory study of proteins that behaved abnormally, in studies of FTLD cases first. What Lee and Trojanowski did not expect was to find a form of TDP-43 in all the ALS cases they subsequently studied.

To identify the protein, the research team first made antibodies to the presumptive misfolded disease protein they believed was responsible, whose identity they didn't know at this stage of their studies. Next, they took brain extracts containing the mystery protein and injected them into mice. The mice then developed the monoclonal antibodies that recognize TDP-43. All 72 cases of FTLD or



John Trojanowski and Virginia Lee

ALS the researchers examined contained misfolded TDP-43.

“Since many cases were studied, the data became very compelling,” recalls Lee, the John H. Ware 3rd Professor in Alzheimer’s Research, professor of pathology and laboratory medicine, and director of the Center for Neurodegenerative Disease Research. Still, in the discussions at the time, other scientists were skeptical that TDP-43 was to blame for the pathology of ALS.

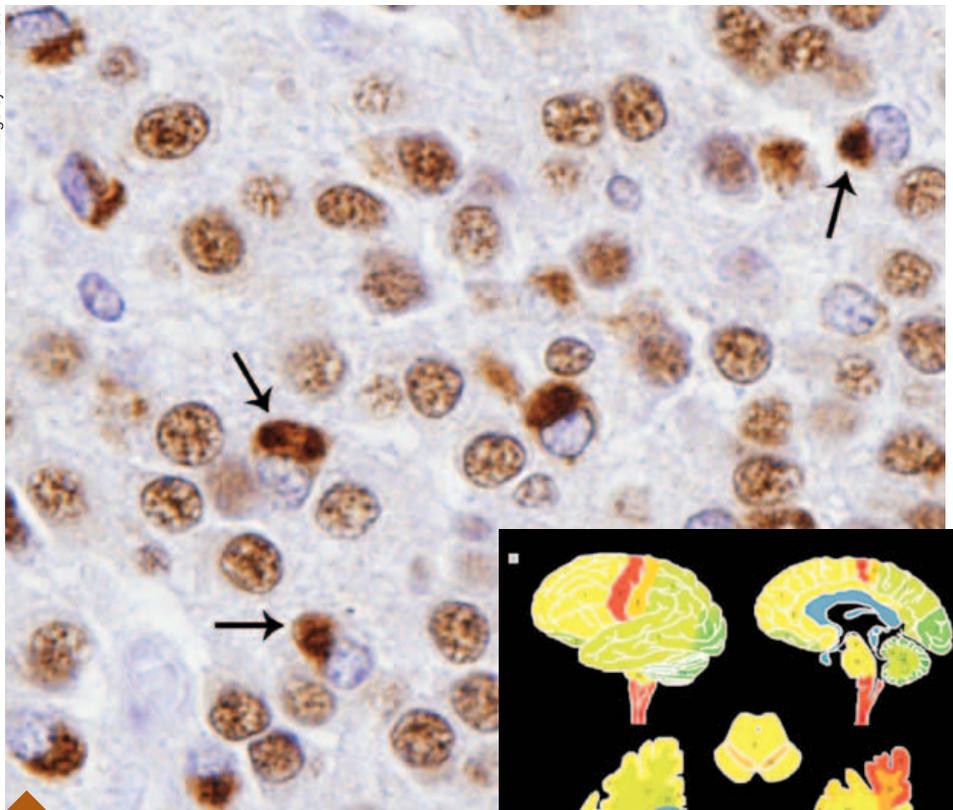
Two years later, however, further proof emerged that TDP-43 is the misfolded protein in ALS and FTLN, through findings that TDP-43 mutations track with the disease. A flurry of reports, including one from Penn, showed that DNA isolated from brain tissue of ALS and FTLN patients harbored mutations in the gene that encodes TDP-43. Penn researchers surveyed 259 individuals either with ALS or with both ALS and FTLN, where misfolded TDP-43 protein was present. The team was also able to determine the DNA sequence of the TDP-43 gene. In addition, the investigators found two families in which a mutation was present. Within the same family, all members who have the disease carry the mutated form of TDP-43. Unaffected individuals lacked the mutation. Other groups made similar findings around the same time, strengthening the evidence.

“When all the mutations began to appear in 2008, investigators who expressed some doubts about our finding were won over,” says Trojanowski, the William Maul Measey-Truman G. Schnabel Jr., M.D., Professor of Geriatric Medicine and Gerontology, professor of pathology and laboratory medicine, and director of the Institute on Aging.

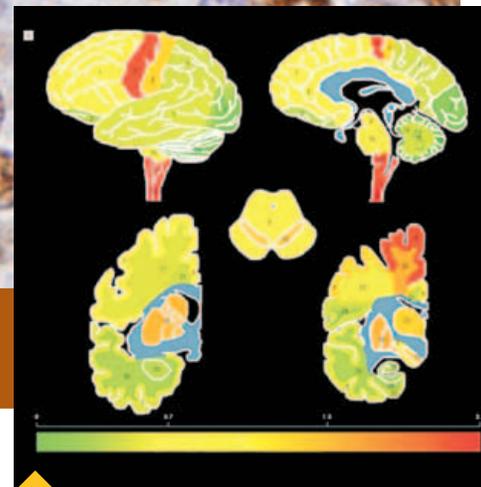
“A paradigm shift”

The emerging field of TDP-43 biology soon underwent a change in understanding how and where the diseases mani-

Image by Felix Geser



The arrows indicate misfolded disease proteins in neurons in the hippocampus.



Red, orange, and yellow depict areas of highest density of TDP-43 pathology.

Image by Felix Geser and Nick Brandner, Archives of Neurology.

festated themselves in the brain. Trojanowski and Lee showed that misfolded TDP-43 accumulates throughout the brain as well as the spinal cord of ALS patients. What that suggests is that ALS has broader neurological effects than scientists previously appreciated.

By again using TDP-43-specific antibodies to examine post-mortem brain tissue of ALS patients, Lee and Trojanowski observed effects in the areas of the brain and spinal cord that control voluntary movements. That was expected, based on the disease’s symptoms. What they did not expect, however, was to see the effects in regions of the brain that involve cognition, executive functioning, memory, and involuntary muscle control.

As initially proposed in 2006, the new evidence supported the idea that ALS, as

ALS-PLUS (ALS with cognitive impairments), and FTLN all had the same underlying molecular pathology involving abnormal TDP-43.

As Trojanowski puts it, “This constituted a paradigm shift in the way we think about these diseases.”

At this point, researchers had firmly established that TDP-43 was the culprit in some cases of ALS and FTLN. They did not yet know how mutated TDP-43 might cause disease or what other genes and proteins played a role. This deepening of inquiry would take research to several different labs at Penn (and elsewhere) and at least three animal models.

More genetic factors that affect TDP-43

During his days as a postdoctoral student at Massachusetts Institute of Technology, Aaron Gitler, Ph.D. '04, used a novel approach to screen for neurodegenerative disease genes. Although the approach was novel, the material was not: yeast cells, the same cells that bakers and brewers have used for centuries to make bread and beer. In the simple yeast cells, misfolded TDP-43 forms clumps just as it does in human nerve cells. The clumping process takes decades to show up in humans but the researchers could model the process within a matter of hours in yeast cells. This advance allows for rapid genetic screening to identify proteins or even drugs that potentially could reverse harmful effects. The next step would be to test the “hits” they found in animal models.

Using a combination of the yeast TDP-43 system and fruit flies, Gitler, then an assistant professor of cell and developmental biology at Penn, and Nancy Bonini, Ph.D., professor of biology in Penn's School of



Photograph by Candace diCarlo

Nancy Bonini

Arts & Sciences and an investigator of the Howard Hughes Medical Institute, found evidence that mutations in the ataxin 2 gene were a genetic contributor to ALS cases associated with TDP-43 abnormalities. More specifically, the study showed that repeats of a bit of DNA that encodes the

amino acid glutamine in the ataxin 2 gene – a genetic stutter, as it were – are associated with an increased risk for ALS. The research began with Gitler's yeast screens in which genes that could suppress or enhance TDP-43 toxicity were identified. The team transferred 5,500 yeast genes into a strain of yeast they had engineered to express misfolded human TDP-43. Among the genes that modified toxicity was the yeast counterpart of ataxin 2.

Gitler and Bonini transferred the genes to fruit flies to assess the effects of the genes and their interactions in the nervous system. When the researchers directed expression of misfolded TDP-43 to the eye of the fruit fly, a progressive, age-dependent degeneration began. When directed to motor neurons, the flies progressively lost the power to move spontaneously. Gitler, Bonini, and researchers at the Center for Neurodegenerative Disease Research then went on to show that people with the same genetic stutter in their ataxin 2 gene had an increased risk for developing ALS. (Gitler has now joined the Stanford School of Medicine.)

But ataxin 2 was not the only gene affecting misfolded TDP-43. Vivianna Van Deerlin, M.D., Ph.D., associate professor of pathology and laboratory medicine at Penn, led an international study using post-mortem brain tissue from 515 patients with frontotemporal lobar degeneration that was associated with TDP-43. The researchers found that these patients had many genetic variations called SNPs in common in a region on chromosome 7 containing the protein TMEM106B. In contrast, in the control group of more than 2,500 disease-free patients, there were no such genetic variations. Based on this finding, the team concluded that the TMEM106B gene variants confer a higher genetic risk for all FTLTDP patients, as well as in the subset of FTLTDP patients with disease-causing mutations in another protein called progranulin.



James Shorter



Virginia Lee consults with Todd Cohen.

Beginning to understand how mutated TDP-43 causes disease

At the same time that some researchers were delving into the genetic evidence linking TDP-43 and disease, James Shorter, Ph.D., assistant professor of biochemistry and biophysics, was studying how TDP-43 misfolds at the protein level. He found that, in the absence of other molecular components, pure normal TDP-43 rapidly assembles into short soluble polymers called oligomers and aggregates. These bear remarkable outward structural resemblance to the aggregates observed in the degenerating motor neurons of ALS patients. As Shorter notes, both normal and mutated TDP-43 form aggregates in his system, and some TDP-43 mutants accelerate aggregation.

In particular, Shorter's laboratory found that a section at one end of TDP-43's amino acid sequence starts the misfolding and aggregation of pure TDP-43. This finding corroborated observations made about yeast by Aaron Gitler.

According to Shorter, it was a chance conversation among Shorter, Gitler, and Oliver King at the Boston Biomedical Research Institute that led to an unexpected twist in the investigation of TDP-43. Using

a sophisticated bioinformatics approach, King recognized that the misfolding-initiating section of TDP-43 is remarkably similar to the type of section that enables some proteins to form prions in yeast. Prions are misfolded proteins that are implicated in mad cow disease in cattle and in Creutzfeldt–Jakob disease in humans. The unexpected conclusion was that vir-

tually all of the mutations in TDP-43 that are linked to ALS lie in the prion-like section of TDP-43.

Shorter's lab then went on to establish that, in the context of the pure protein, some ALS-TDP-43 mutations can accelerate aggregation, whereas other mutations do not. These findings meshed with other observations made by Gitler's group in yeast, in which some ALS-linked TDP-43 mutations promote aggregation and toxicity, whereas others do not and result in proteins that are very similar to normal TDP-43. These data suggested that there is more than one way by which mutations promote ALS.

Shorter's team is now investigating methods to prevent or reverse the misfolding of TDP-43. Shorter notes, "The powerful combination of our pure protein biochemistry and Aaron Gitler's approaches in yeast is likely to yield many new and profound insights into ALS, which will undoubtedly change the way we think about this disease."

A later chapter in the TDP-43 story added yet another wrinkle: Virginia Lee



In the Center for Neurodegenerative Disease Research, slides of brain tissue.

showed in a mouse model the first direct evidence of how mutated TDP-43 can cause neurons to die. When human mutated-TDP-43 genes are put into mice, the mouse nerve cells die because they stop producing enough normal mouse TDP-43. Because cells regulate the exact amount of TDP-43, over-expression of the human TDP-43 protein prevents the mouse TDP-43 from functioning normally.

In Lee's view, this effect leads to neuron death in this model rather than clumps of TDP-43 because these clumps were rare in the mouse cells observed in this study. She says that it is not yet clear why clumps were rare in these mice but so prevalent in human post-mortem brain tissue of ALS and FTLN patients.

The researchers are now back to looking for more genetic partners for TDP-43-specific genes that are regulated by TDP-43 and trying to discover how messenger RNA (mRNA) is involved. In addition to other functions, TDP-43 stabilizes the structure of mRNA.

Knowing the genes involved in the normal function of TDP-43 will help researchers identify what goes awry when normal TDP-43 is missing or nonfunctional or when clumps of misfolded TDP-43 crowd a cell's interior.

The continuing work on animal models is about to bear fruit as well: Five years after the publication of the original paper on TDP-43, Lee notes that the center she directs will soon launch studies of strategies to prevent TDP-43-mediated degeneration of the nervous system using this mouse model of TDP-associated amyotrophic lateral sclerosis and frontotemporal lobar degeneration.

..... **An Epilogue – But Not the Last Word**

A major hurdle in understanding neurodegenerative diseases is establishing a clear picture of what leads up to neuron death. In recent years, much data have

been published indicating that increased oxidative stress plays a role in the degeneration and death of neurons. Oxidative stress is an imbalance between the production of reactive oxygen molecules and the body's ability to get rid of them. Disturbances in the normal oxygen state of tissues can damage all components of the cell, including proteins and DNA.

Most recently, Todd Cohen, Ph.D., a postdoctoral fellow in the Lee lab, studied how TDP-43 reacts to oxidative stress. His work was published in the *EMBO Journal* in December. Stress induces the protein to move from the nucleus to the cell cytoplasm; its ability to fold properly is altered as well. Then, in January, Lee, Trojanowski, and Edward B. Lee, assistant professor of pathology and laboratory medicine, published a related review of TDP-43-mediated neurodegeneration in *Nature Reviews Neuroscience*. There, they suggest two reasons for the neurodegeneration. It could result from the protein's loss of function as it misfolds and is no longer available to regulate gene expression; or it could result from a gain of toxic properties as it forms clumps in the cell cytoplasm, disrupting normal day-to-day functions.

In the *EMBO Journal*, Cohen explained how TDP-43 reacts to stress chemically and showed that it is reversible. His team found that oxidizing chemicals caused cross-linking between sulfur molecules of the amino acid cysteine in the TDP-43 proteins in culture. This linking caused the protein to misfold. In addition, the researchers also saw sulfur-sulfur bonding between two or more proteins, which was the start of debilitating TDP-43 clumps.

What was more surprising was that they were able to break up the TDP-43

clumps in the cultured cells and in post-mortem human brain tissue samples from FTLN-TDP patients. They also showed that these disaggregated proteins became functional again.

The "big take-home message," says Cohen, is that antioxidant therapy – particularly a well-known one called NAC – could prevent the sulfur cross-linking in the first place. That would prevent the protein misfolding and the multi-protein clumps seen in many neurodegenerative diseases. According to Cohen, future studies – first in animal models and



Edward Lee

eventually in humans – could evaluate whether taking NAC or related antioxidant supplements could be an effective treatment strategy to prevent amyotrophic lateral sclerosis or frontotemporal lobar degeneration.

There appears to be more and more to this saga of TDP-43, a single protein that plays some very important roles. The way scientists are producing data around TDP-43 and many neurodegenerative diseases suggests that the collective research effort is beginning to gain momentum. ♥