The Blazeman Foundation for ALS Fellowship Progress Report April 2010 – March 2011

The Division of Neuromuscular Medicine, Neurogenetics Laboratory
Davee Department of Neurology and Clinical Neurosciences
Northwestern University Feinberg School of Medicine

This funding year we focused our studies on two new genes related to ALS, the first, Optineurin, where mutations have been recently linked to ALS and the second gene is XALS gene, where mutations were linked to ALS by genetic analysis in our Laboratory.

These two new genes are of great interest in understanding the mechanism of ALS because we have found that their protein product was present in a wide spectrum of ALS cases, strongly suggesting a pathogenic role beyond the genetic mutations found. With these new discoveries, we have advanced in forging a more unified pathogenic pathway shared between sporadic ALS (SALS) and several types of familial ALS (FALS) and some other neurodegenerative diseases.

1- Differential involvement of Optineurin in amyotrophic lateral sclerosis with or without *SOD1* mutations:

This study was conducted in order to determine if optineurin-positive inclusions are common pathogenic feature in SALS and FALS, including SOD1-linked ALS. We analyzed 50 postmortem spinal cord samples from cases with various type of ALS using immunostaining and western bloting. Spinal cord sections from controls without ALS were also analyzed.

We found optineurin was present in skein-like inclusions in all examined spinal cord of SALS and FALS cases with the only exceptions being cases with SOD1 mutations and transgenic mice over-expressing G93A SOD1 mutations. The specificity of the immunostaining using antibodies to optineurin was confirmed with western blot, where a major single band corresponding to molecular weight of optineurin was detected (figure1).

Our results showed that optineurin is involved in the pathogenesis of SALS and FALS without SOD1 mutations. We have previously shown that non SOD1 ALS also have TDP43 and FUS pathology (Mackenzie et al, 2007 and Deng et al, 2010). The presence of skein-like inclusions in most of the non-optineurin-linked ALS cases studied, support the notion that optineurin, along with TDP43 and FUS, participates in the pathological inclusions noticed in ALS.

This work, in which I was one of the contributors, through the Blazeman foundation, was submitted and accepted in Archive of Neurology. It's now in press.

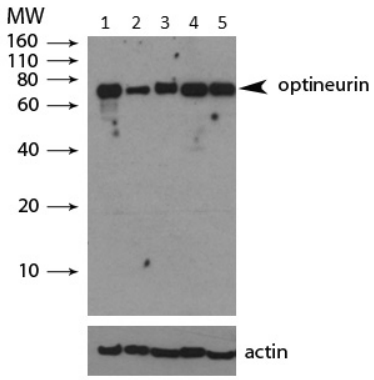


Figure 1: Western blots with antibody to optineurin. Lane 1, FALS; Lanes 2-4, SALS; Lane 5, Duchenne's muscular dystrophy. A single optineurin band of the expected size detected is indicated by an arrow. Molecular weight (MW) is indicated on the left in kilodaltons. Lower panel, actin control.

2- Mutations in a new gene cause dominant X-Linked form of Juvenile and Adult-Onset ALS and ALS/dementia:

Mutations in a gene causing X-linked ALS and ALS/dementia patients have been previously identified in our laboratory. These mutations caused abnormal accumulation of the XALS protein in the motor neurons of the brain, including hippocampus and cortex, and in the anterior horns of spinal cord.

We tested the hypothesis that XALS protein is also involved in the pathology of other forms of ALS. Using immunohistochemistry, e XALS protein, was found in the skein-like inclusions in virtually all type of ALS, including SALS, ALS with dementia, FALS with G298S mutation in TDP43, FALS without SOD1, TDP43 and FUS mutations. It wasn't detected in SOD1-linked ALS. These studies suggest a pathogenic role of XALS in neuronal degeneration. Thus XALS protein, along with FUS, TDP43 and optineurin are partners in a common downstream pathway in the degeneration of motor neuron. This appears to be different from SOD1 linked neurodegeneration. These finding are novel in terms of pathogenicity and localization of the XALS protein aggregates in the brain and spinal cord.

Figure 2, shows various kind of ALS which are associated with XALS, P62, optineurin, TDP43 and FUS proteins and summarize the common downstream pathway involved in ALS.

This major work involved a large group of scientists and years of research, was finally submitted to Nature in December 2010 and it's under review process. My contribution was to determine the antibodies specificity to XALS protein and collaboration in co-localization analysis with other ALS Proteins such as TDP43 and FUS. We are in the process of addressing the reviewer's critiques to finalize the paper.

<u>- Characterization of panel of antibodies to XALS protein:</u>

Antibodies to different regions of XALS protein, generated previously in the laboratory were purified using peptide affinity purification (Table1). The specificity of the antibodies was determined either by western blot or Immunohistochemistry (IHC) (Figure 3,4)

- Generation of a recombinant human protein homologous to XALS (hPHX):

Since hPHX shares up to 70% homology with the XALS protein, we generated such a protein, using N2A expression system and used as control to detect any cross-reactivity of XALS antibodies with this protein.

- Co-localization analysis of XALS and TDP43:

TDP43 wild type or truncated form (C-terminal 195AA fragments) tagged to cherry (generated in 2009-2010) were transfected with XALS wild type and mutant in N2A cells (Mouse neuroblastoma). Only the 195AA TDP43 fragment showed co-localization with XALS wild type or mutant, suggesting an involvement of XALS and TDP43 in a common pathological pathway (Figure5). Further experiments are required to confirm this promising hypothesis.

- Co-localization analysis of XALS and FUS:

FUS wild type and ALS mutant R495X tagged to GFP and Myc (generated in 2009-2010) were co-transfected with XALS wild type and mutants in N2A cells (Mouse neuroblastoma). We did not find any co-localization of Wild type XALS or mutant with FUS wild type or mutant FUS R495X which translocate from the nucleus to the cytoplasm. (Figure6, modified from Deng et al, 2010)

Figure 2, shows various kind of ALS which are associated with XALS, P62, optineurin, TDP43 and FUS proteins and summarize the downstream pathways involved in ALS.

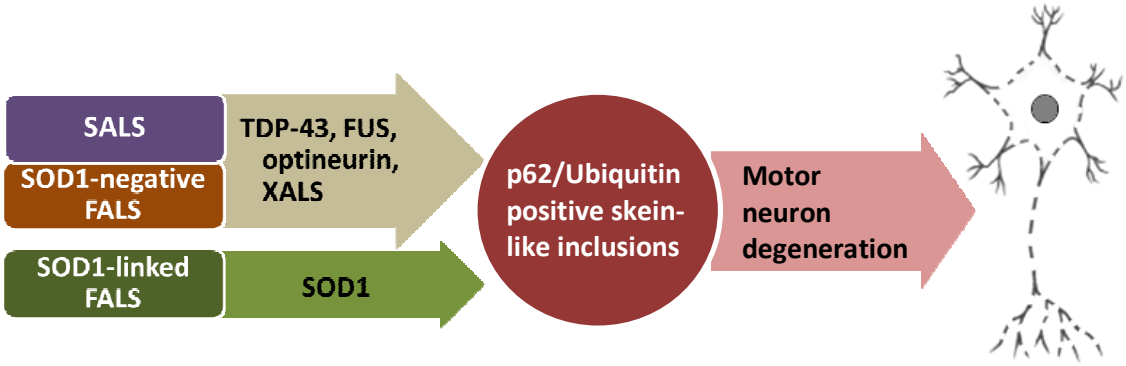


Figure2: Schematic model depicting two parallel pathways involved in the pathogenesis of ALS.

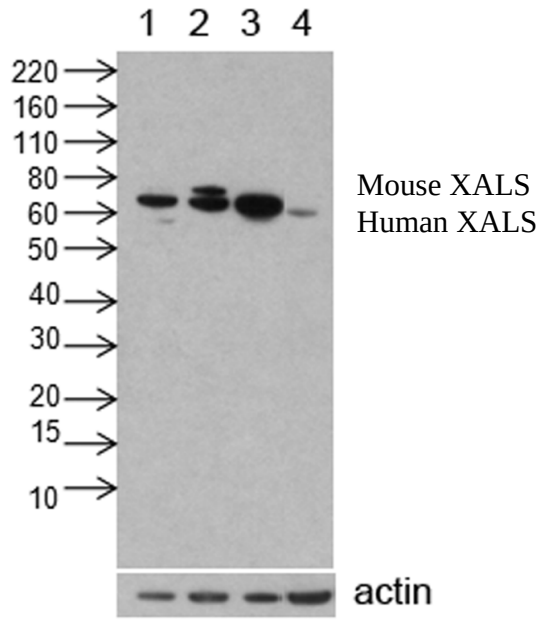


Figure 3: Western blot with N-antibody to XALS. Human XALS-transfected SH-SY5Y (lane 1), neuro-2a (lane 2) and HEK293 cells (lane 3) and untransfected HEK293 cells. Mouse XALS and human XALS are indicated. Lower panel, actin control. Molecular weight is shown on the left in kilodaltons.

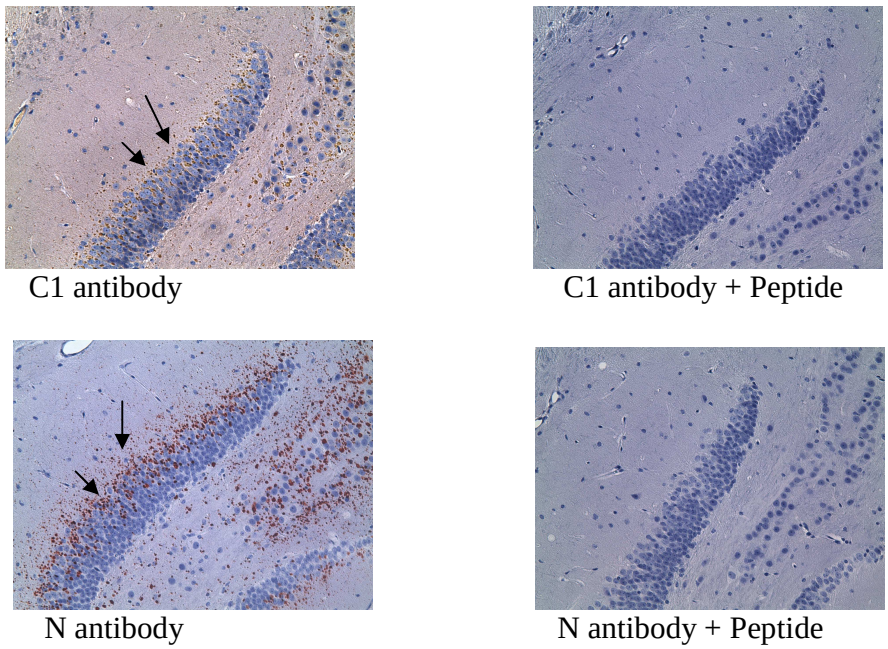
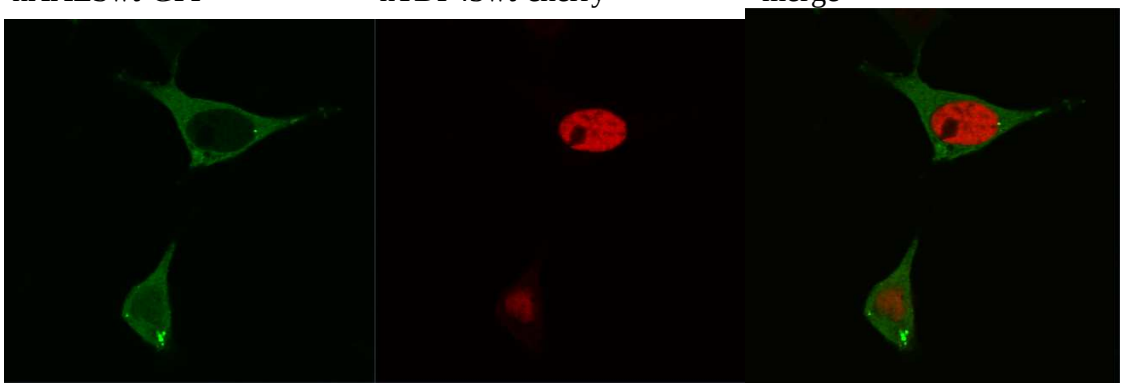


Figure 4: C1 and N antibodies immunohistochemistry staining of mutant P497/H XALS transgenic mouse hippocampus region. Absence of aggregates staining in the slides treated with antibody and its peptide at 1ug/ml. Arrows show XALS mutant aggregates.

A

hXALSwt-GFP hTDP43wt-cherry merge

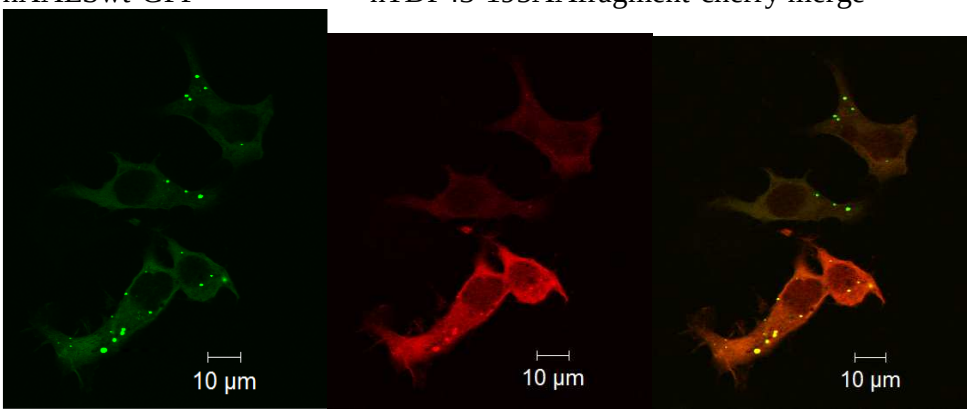


B

hXALSmutP497/H-GFP hTDP43-cherry merge

C

hXALSwt-GFP hTDP43-195AAfragment-cherry merge



D

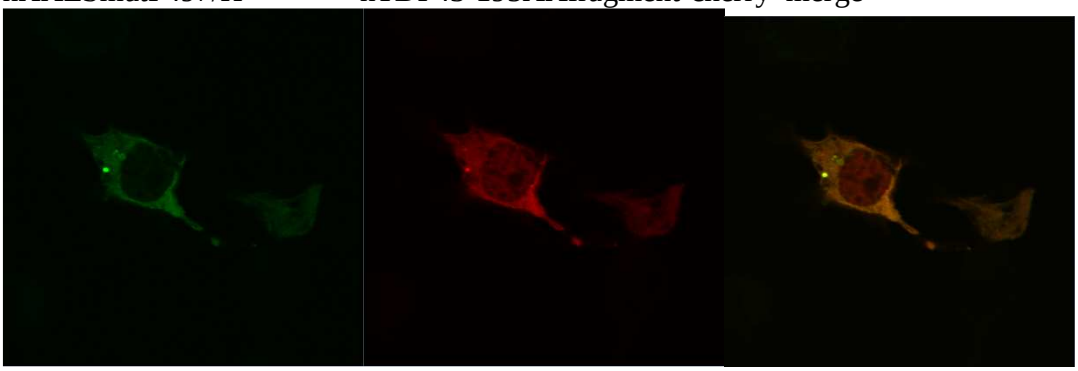
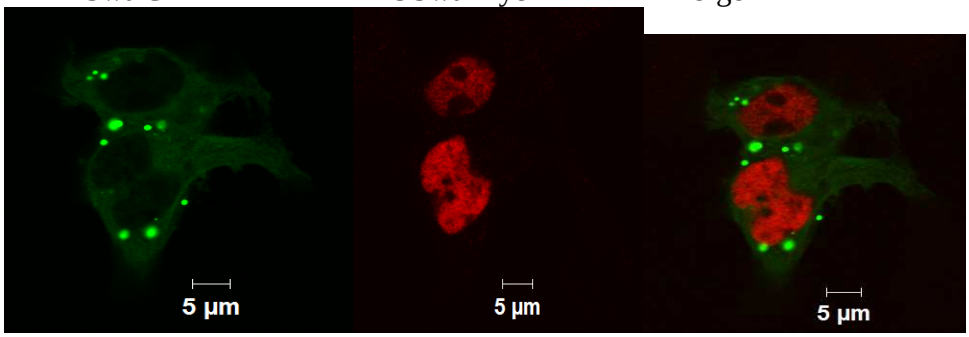
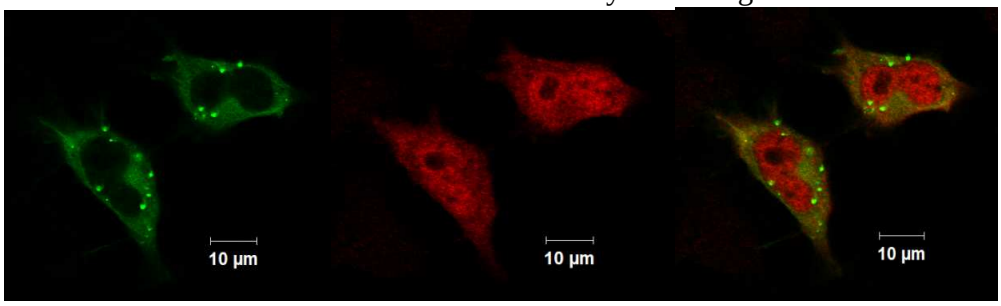


Figure5: TDP43 co-localize with XALS wild type or mutant P497/H when it is translocated to the cytoplasm in its truncated form, TDP43 C-terminal 195AA fragment, suggesting an involvement of XALS and TDP43 in a common pathological pathway.

A

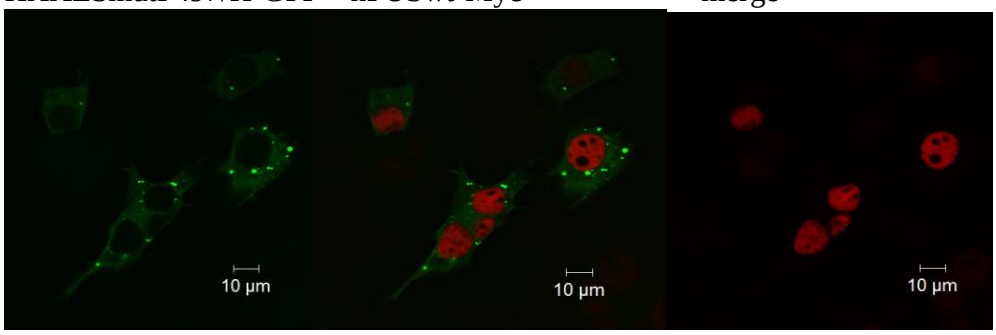


B



C

HXALSmutP497/H-GFP hFUSwt-Myc merge



D

hXALSmutR497/H-GFP hFUSmutR497/X-Myc merge

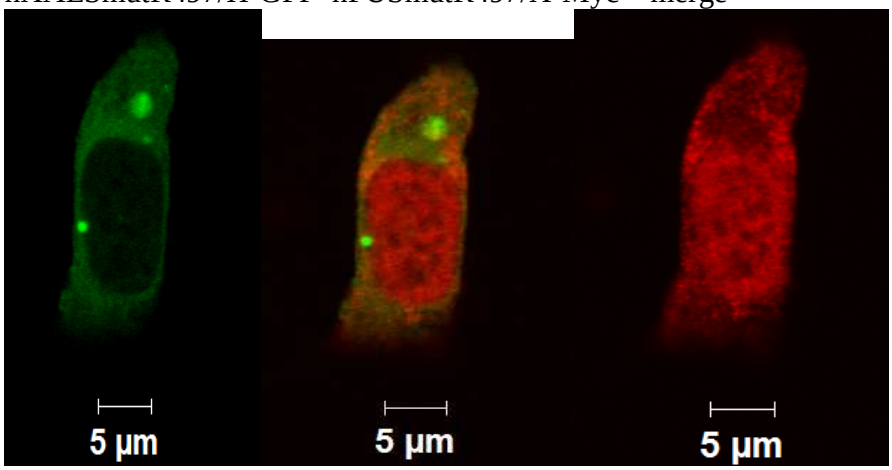


Figure6: No evidence of co-localization between human XALS wild type or mutant and FUS wild type or mutant.

Table1: Panel of antibodies against hXALS and applications:

Antibody	WB(mouse tissue)	IHC (mouse tissue)	IHC (human tissue)
N1 (N-terminal 7-25AA)	good	Good, specific	good
S (Stitch domain 115-133AA)	multibands	good, KO+	
C1 (C-terminal 508-533AA)	multibands	good, specific	good
C2 (C-terminal 565-579AA)	Multibands	good	
Commercial (C-terminal last 50AA)	good	good	good

3-Stem Cell Research project:

While we are optimizing the conditions to reprogram the human fibroblast with recombinant proteins, Oct4, Sox2, cMyc and Klf4, generated from HEK293 stable expression systems, we are setting up a lentivirus reprogramming strategy that will enable us to move faster in the generation of iPSCs. This lentivirus (STEMCCA, Millipore) is more efficient in delivering and reprogramming of proteins. This lentivirus expresses four transcription factors (Oct4, Sox2, cMyc and Klf4) in a single polycistronic cassette which means, the four genes, are contained in one viral vector. The viral vector is an excisable policistronic viral vector; the genes will be incorporated into the host chromosome and then excised using the Cre excision system. This feature results in significant reduction of the risks related to spontaneous mutations of the cell host chromosomes, which is a major issue in transforming iPSCs in to neurons.