

Award Number: W81XWH-11-1-0449

TITLE: Hypermetabolism as a Risk Factor for ALS

PRINCIPAL INVESTIGATOR: Philip C. Wong, Ph.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University  
Baltimore, MD 21218

REPORT DATE: July 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*  
*OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

<b>1. REPORT DATE</b> 01-07-2012		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 1 Jul 2011 - 30 Jun 2012	
<b>4. TITLE AND SUBTITLE</b> Hypermetabolism as a Risk Factor for ALS				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-11-1-0449	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Philip C. Wong, Ph.D.  E-Mail: wong@jhmi.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  The Johns Hopkins University  Baltimore, Maryland 21205				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> ALS, a fatal adult-onset disease, is a neurodegenerative disorder that affects motor neurons in the brain and the spinal cord. What causes ALS remains incompletely understood and no disease modifying therapy is currently available. Recent discovery of an ALS gene called <i>Tat Activating Regulatory DNA Binding Protein (TDP-43)</i> encoding a protein involved in RNA metabolism, provided opportunities to clarify disease pathogenesis and hold promise for development of new therapies. Our recent discovery in mice that lack <i>Tdp-43</i> showed that these mice excessively burned out their body fat, likely working through a protein called <i>Tbc1d1</i> which is known to control fat metabolism in skeletal muscle. To test this hypothesis, we generated conditional <i>Tdp-43</i> knockout mice using a muscle-specific (MLC) driver of Cre recombinase. Loss of <i>Tdp-43</i> in skeletal muscle led to adult onset weight loss beginning about 3 months of age followed by premature death within a month. Pathological examination of skeletal muscles revealed degeneration of fibers as well as marked regeneration, observations that are consistent with a myopathy occurring in these mice. These new findings suggest an important role for <i>Tdp-43</i> in skeletal muscle that may contribute to the pathogenesis of ALS and possibly a select group of myopathies.					
<b>15. SUBJECT TERMS</b> Tat Activating Regulatory DNA Binding Protein (TDP-43), conditional knockout mice, fat metabolism, skeletal muscle, myopathy					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  UU	<b>18. NUMBER OF PAGES</b>  9	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U			<b>19b. TELEPHONE NUMBER</b> (include area code)

## Table of Contents

	<u>Page</u>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4</b>
<b>Key Research Accomplishments.....</b>	<b>8</b>
<b>Reportable Outcomes.....</b>	<b>8</b>
<b>Conclusion.....</b>	<b>8</b>
<b>References.....</b>	<b>8</b>
<b>Appendices.....</b>	<b>9</b>

## Introduction

Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disorder characterized by the progressive loss of motor neurons in the brain and spinal cord, is fatal and at present, there is no effective mechanism-based therapy. Our previous work indicated that hypermetabolism related to loss of TDP-43, an essential RNA binding protein implicated in ALS and Frontotemporal dementia (FTD), may contribute to disease pathogenesis (1). Specifically, we generated a conditional *Tardbp* knockout mouse model to determine the physiological role of TDP-43 in the adult animal. Surprisingly, we found that post-natal deletion of *Tardbp* in mice caused dramatic loss of body fat (1). We discovered one downstream targets of TDP-43, termed *Tbc1d1*, a gene primarily expressed in skeletal muscle and known to regulate body fat metabolism (2) and linked to obesity (3), and is down-regulated in the absence of TDP-43 (1). Thus, our results implicate the role of *Tardbp*-dependent *Tbc1d1* in skeletal muscle to regulate body fat metabolism. That some individuals with ALS showed a hypermetabolic state (4) and that there is evidence for defective energy homeostasis in ALS (5) raised the possibility that hypermetabolism may contribute to the pathogenesis of ALS. Although it is not clear how hypermetabolism influences pathogenesis of ALS, it will be of great interest to test directly in mouse models of ALS whether altered fat metabolism participates in motor neuron degeneration using our conditional *Tardbp* knockout mouse model that exhibit a lean phenotype. Positive outcomes from these studies will offer the possibility that dietary or nutritional interventions to attenuate excessive fat metabolism would be beneficial for patients with ALS. This is particularly important as the nutritional status is thought to be a prognostic factor for survival in ALS (6) and that personalized nutritional management of patients may be a beneficial strategy for symptomatic treatment of ALS (7). Moreover, strategies to up-regulate *Tbc1d1* in skeletal muscles may also be an attractive alternative approach towards development of mechanism-based therapies for ALS. Thus, our proposed studies will have direct impact on the development of therapeutics that can be translated into disease-modifying treatments for this devastating disease of the elderly. Because TDP-43 inclusions has been observed in a variety of other neurodegenerative diseases (reviewed in 8), such as Inclusion Body Myopathy associated with Paget's disease of bone and FTD (IBMPFD), it will also be interesting to test whether hypermetabolism may also be a risk factor in these illnesses. If so, our work will have important therapeutic implications beyond ALS.

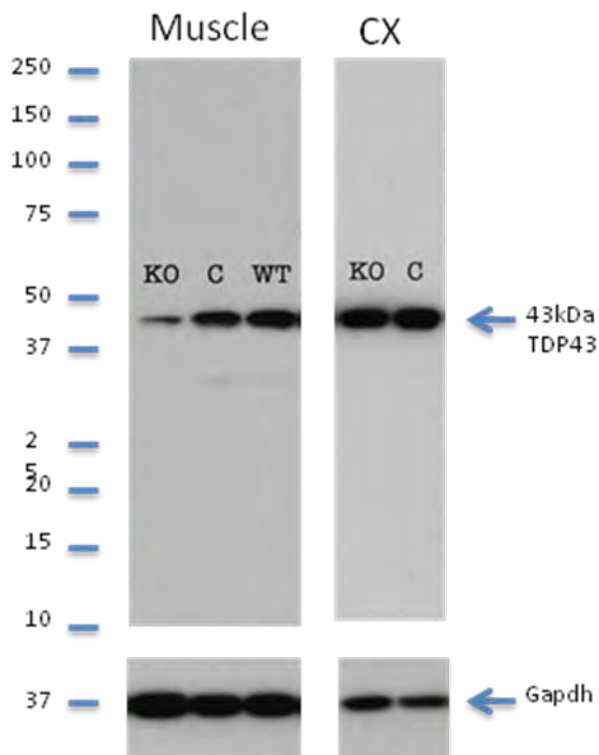
## Body

### 1. Loss of Tdp-43 in skeletal muscle leads to adult onset weight loss and premature death

To obtain mice lacking Tdp-43 exclusively in skeletal muscle, we crossbred *Tdp-43<sup>F/F</sup>* mice with *MLC-Cre;Tdp-43<sup>+/-</sup>* mice. Mice expressing Cre recombinase driven by regulatory sequences of the Myosin Light Chain 1/3 locus (MLC) demonstrated Cre activity in only skeletal muscle fibers, specifically in fast twitch (Type II) but not in slow twitch (Type I) fibers. Expected Mendelian ratios were observed from these crosses (Table 1), suggesting that Tdp-43 is dispensable in skeletal muscle during embryonic development.

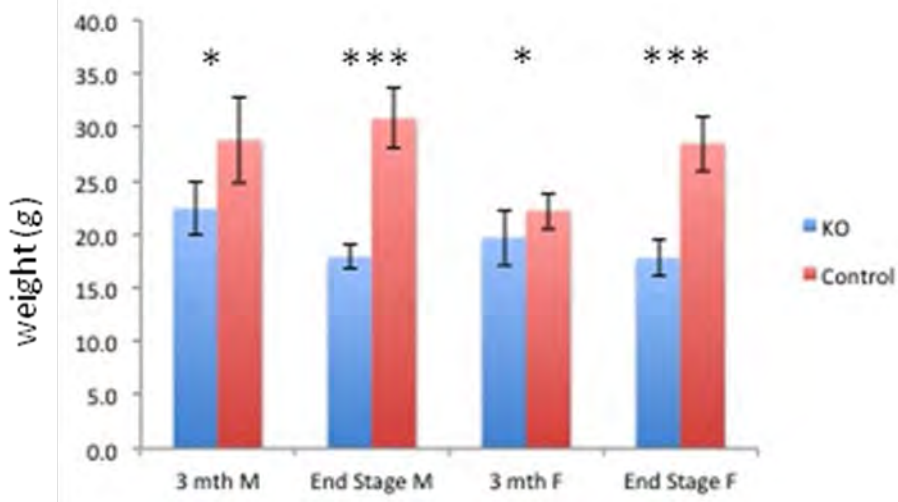
	MLC-Cre; Tdp-43 +/-			
Tdp-43 F/-	MLC-Cre;Tdp-43 F/+ 1/8	MLC-Cre;Tdp-43 F/- 1/8	MLC-Cre;Tdp-43+/- 1/8	MLC-Cre;Tdp-43-/- 1/8 (embryonic lethal)
	Tdp-43 F/+ 1/8	Tdp-43 F/- 1/8	Tdp-43 +/- 1/8	Tdp-43 -/- 1/8 (embryonic lethal)

**Table 1:** Progeny from crosses between Tdp-43 F/- and MLC-Cre;Tdp-43+/- mice yielded expected Mendelian frequencies for all genotypes.



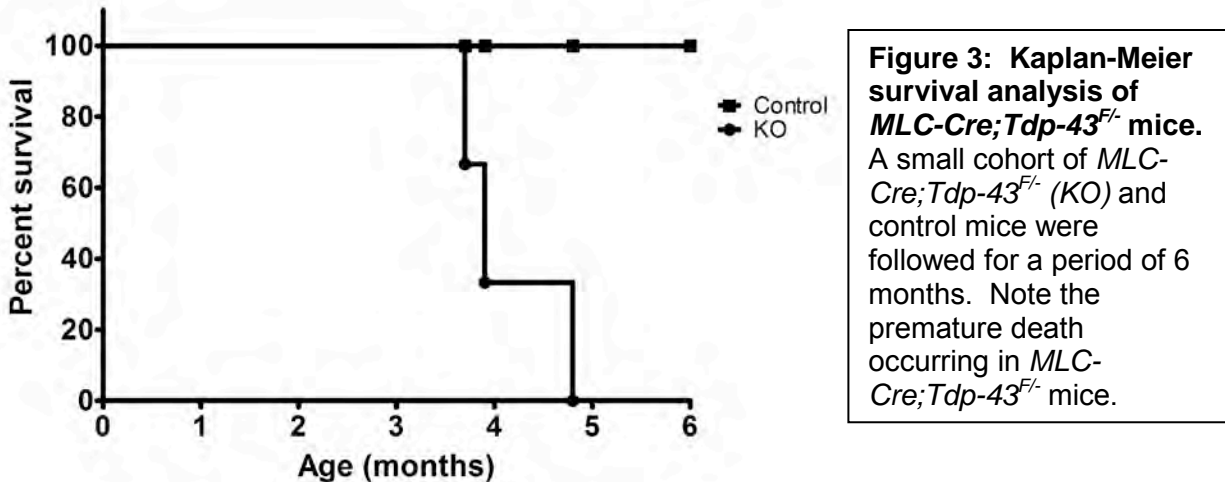
**Figure 1: Reduction of Tdp-43 in skeletal muscle of *MLC-Cre;Tdp-43<sup>F/F</sup>* mice.** Equal amount of protein extracts from skeletal muscle (left panel) and brain (CX; right panel) were immunoblotted with antisera against Tdp-43. Blots were re-probed with antisera against Gapdh to control for loading. Molecular weight markers (in kD) are indicated on the left. KO= *MLC-Cre;Tdp-43<sup>F/F</sup>* mice, C= *MLC-Cre;Tdp-43<sup>+/-</sup>* mice, WT=wild type mice. Note the marked reduction of Tdp-43 in skeletal muscle of *MLC-Cre;Tdp-43<sup>F/F</sup>* mice.

Protein expression of Tdp-43 was substantially reduced in skeletal muscle of *MLC-Cre;Tdp-43<sup>F/F</sup>* mice (Fig. 1, left panel), but remained unaffected in brain (Fig. 1, right panel) cardiac as well as smooth muscle (data not shown). Levels of Tdp-43 were consistent with that of control mice in a variety of other tissues sampled. Both *MLC-Cre;Tdp-43<sup>F/F</sup>* mice and control mice showed comparable growth and development into early adulthood. However, from about 3 months of age, mice lacking Tdp-43 in skeletal muscles decline in weight and show dramatic reduction in body size within a month (Fig. 2). These mice reach end stage within 6 weeks after noticeable onset of weight loss. End stage in *MLC-Cre;Tdp-43<sup>F/F</sup>* mice is characterized by an emaciated appearance, a pronounced hunch, inactivity and labored breathing. Kaplan-Meier analysis of a small cohort of mice indicates an average survival of 4 months for *MLC-Cre;Tdp-43<sup>F/F</sup>* mice while control littermates survived the span of the experiment (Fig. 3).



**Figure 2: Weight loss in *MLC-Cre;Tdp-43<sup>F/F</sup>* mice.** Weight of mice were monitored over a 4 month period. Note that *MLC-Cre;Tdp-43<sup>F/F</sup>* mice showed marked weight loss near end stage.

These mice were also monitored for muscle strength using the hanging wire test. Mice were suspended from a wire grid and their latency to fall was recorded with a maximum of 60 seconds. The test was carried out every 2 weeks beginning at about 6 weeks of age. There was no appreciable difference in the hang time of *Tdp-43* knockout and control mice even at 4 months (data not shown), although this could be confounded by the marked decrease in weights of *MLC-Cre;Tdp-43<sup>F/-</sup>* mice as they progressed towards end stage. We also noted that *Tdp-43* knockout mice were less active and did not move around as much on the wire grid.



**Figure 3: Kaplan-Meier survival analysis of *MLC-Cre;Tdp-43<sup>F/-</sup>* mice.** A small cohort of *MLC-Cre;Tdp-43<sup>F/-</sup>* (KO) and control mice were followed for a period of 6 months. Note the premature death occurring in *MLC-Cre;Tdp-43<sup>F/-</sup>* mice.

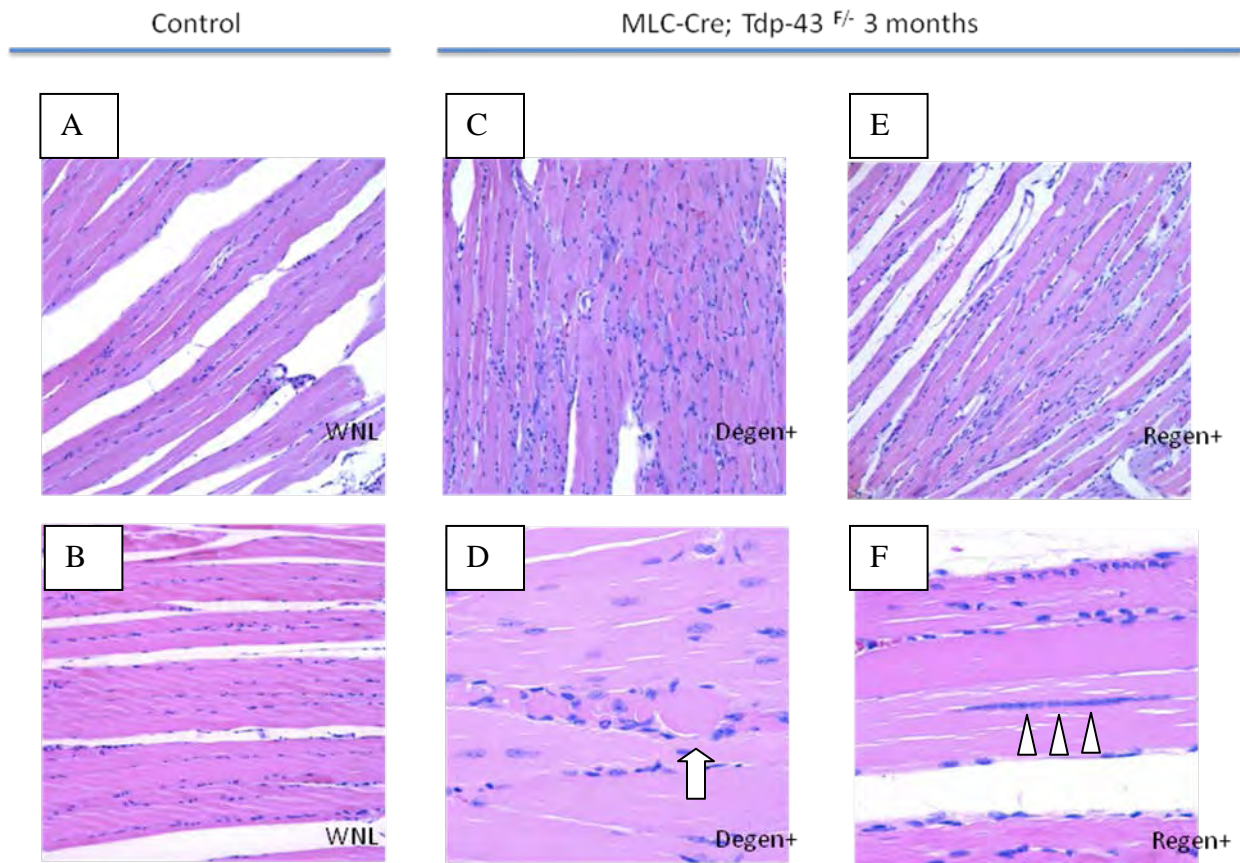
This group of mice was also assessed for performance on the accelerating Rotarod test. *MLC-Cre;Tdp-43<sup>F/-</sup>* mice had comparable average latencies compared to control mice (data not shown), suggesting that there was no deficit in motor coordination.

2. Mice lacking Tdp-43 in skeletal muscle develops a myopathy characterized by degeneration and regeneration of muscle fibers

Gross dissection of mice lacking Tdp-43 in skeletal muscle at end stage show reduced muscle tone, suggestive of muscle atrophy. These mice had very little body fat compared to control littermates (data not shown). At end stage, knockout mice also had small thymuses, with other major organs appearing grossly normal (data not shown).

Pathological analyses of *MLC-Cre;Tdp-43<sup>F/-</sup>* mice at end stage show a striking phenotype that is consistent with myopathy of the skeletal muscles (Fig. 4); with little evidence of histological abnormalities in the other organs, including but not limited to the heart, blood vessels and gastrointestinal tract. Muscle samples taken from the cheek, tongue, quadriceps, diaphragm and sternum of *Tdp-43* knockout mice showed hypercellularity and large variability in fiber size (data not shown). There was muscle degeneration as evidenced by fibers with loss of striation and hyper-eosinophilic rounded fibers (Fig. 4C, D), as well as increased numbers of small atrophic myofibers (Fig. 4D). Importantly, there was also marked regeneration of muscle, as indicated by the large numbers of atypical, enlarged and pale staining (H&E) nuclei in basophilic fibers (Fig. 4E, F). The large number of central nuclei and internal nuclei in rows without basophilia (Fig. 4F) suggests that loss of Tdp-43 may have triggered aberrant developmental programs that may have arrested prematurely. Several foci of perivascular inflammatory cells were also observed in the subcutaneous fat layer of the leg in *MLC-Cre;Tdp-43<sup>F/-</sup>* mice (data not shown). In contrast to end-stage mice, preliminary analyses of younger *MLC-Cre;Tdp-43<sup>F/-</sup>* mice showed that the myopathy preceded the decline in weight. Additional

studies are currently ongoing to determine the molecular pathways underlie the myopathy occurring in these mice lacking Tdp-43 in skeletal muscle.



**Figure 4: Pathological analysis of myopathy in *MLC-Cre;Tdp-43<sup>F/-</sup>* mice.** H & E staining of skeletal muscle from *MLC-Cre;Tdp-43<sup>F/-</sup>* mice (C-F) and age-matched littermate control (A-B). Note degenerating fiber evidenced by loss of striation and hyper-eosinophilic rounded fibers in panel D (arrow) and regenerating fiber characterized by central nuclei and internal nuclei in rows in panel F (arrowheads).

### 3. No apparent effects in mutant dynactin mice crossbred to mutant *Tbc1d1* mice

To address the question as to whether hypermetabolism exacerbates motor neuron disease by assessing the effect of mutant *Tbc1d1* on the onset and survival of mutant *dynactin* mice, we performed a pilot study with a cohort of mice carrying both mutant dynactin and mutant *Tbc1d1*. However, we did not observe any affect in terms of disease onset or survival of compound mutant mice (mice carrying both mutant dynactin and mutant *Tbc1d1*) as compared to the mutant dynactin mice. For this reason, we will not further pursue this line of investigation and focus attention on our unexpected findings of a striking myopathy observed in *MLC-Cre;Tdp-43<sup>F/-</sup>* mice which we hypothesize may have important implications towards our understanding of muscle pathology occurring in human myopathy disease, such as IBMPFD.

## Key Research Accomplishments

- To address the role of Tdp-43 in skeletal muscle, we generated a Tdp-43 mouse model that lack Tdp-43 in specifically in skeletal muscle.
- Diminished amount of Tdp-43 in skeletal muscle led to adult onset weight loss beginning at ~3 months of age.
- Diminished amount of Tdp-43 in skeletal muscle caused premature death at ~4 months of age.
- Pathological analysis of skeletal muscle in mice lacking Tdp-43 in skeletal muscle revealed a myopathy characterized by degeneration and atrophy of muscle fibers.
- These results indicate that Tdp-43 plays an important physiological role in skeletal muscle and implicate the participation of Tdp-43 in the pathogenesis of human myopathies with TDP-43 proteinopathy, such as IBMPFD.

## Reportable Outcomes

Abstract and presentation of poster by Ms. Sophie Lin, (Graduate Student) at the annual Pathology Young Investigator Day at Johns Hopkins in May, 2012 (See Appendices)

## Conclusion

Our investigation into the role of Tdp-43 in skeletal muscle has provided unexpected results that will have profound implications for human myopathies, including IBMPFD. We believe we have created a mouse model of myopathy related to Tdp-43 proteinopathy that will be instrumental in unraveling the pathogenic mechanisms underlying diseases such as IBM or IBMPFD in the future.

## References

1. Chiang P-M, Ling J, Jeong YH, PriceDL, Aja S and Wong PC: Deletion of *Tardbp* Down-Regulates *Tbc1d1*, a Gene Linked to Obesity, and Alters Body Fat Metabolism. *Proc Natl Acad Sci U S A*, online Jul 26, 2010.
2. Chadt A, *et al*: *Tbc1d1* mutation in lean mouse strain confers leanness and protects from diet-induced obesity. *Nat Genet* 40(11):1354-1359, 2008.
3. Stone S, *et al*: TBC1D1 is a candidate for a severe obesity gene and evidence for a gene/gene interaction in obesity predisposition. *Hum Mol Genet* 15(18):2709-2720, 2006.
4. Bouteloup C, *et al*: Hypermetabolism in ALS patients: an early and persistent phenomenon. *J Neurol.* 256:1236-42, 2009.

5. Dupuis L, Oudart H, Rene F, Gonzalez de Aguilar JL, & Loeffler JP: Evidence for defective energy homeostasis in amyotrophic lateral sclerosis: benefit of a high-energy diet in a transgenic mouse model. *Proc Natl Acad Sci U S A* 101:11159-11164, 2004.
6. Desport JC, Preux PM, Truong TC, Vallat JM, Sautereau D, Couratier P.: Nutritional status is a prognostic factor for survival in ALS patients. *Neurology* 53:1059-63, 1999.
7. Cameron A, Rosenfeld J.: Nutritional issues and supplements in amyotrophic lateral sclerosis and other neurodegenerative disorders. *Curr Opin Clin Nutr Metab Care* 6:631-43, 2002.
8. Lagier-Tourenne C, Polymenidou M, Cleveland, DW: TDP-43 and FUS/TLS: emerging roles in RNA processing and Neurodegeneration. *Human Molecular Genetics* 19:R46-R64, 2010.

## Appendices

### ABSTRACT

TAR DNA Binding Protein 43 (TDP-43), an essential RNA binding protein, is thought to play an important role in the modulation of gene expression through regulation of transcription, splicing, and stabilization of mRNA. TDP-43 immunoreactive inclusions have been reported in many neurodegenerative diseases, and misregulation of TDP-43 may contribute to disease pathogenesis either through toxic gain-of-function or loss-of-function mechanisms. Discerning between the two has important ramifications for the design of therapeutic strategies, and to achieve this end, our lab generated a conditional *Tdp-43* knockout mouse model using the Cre-loxP system. Constitutive knockouts of *Tdp-43* are embryonic lethal and postnatal deletion of *Tdp-43* resulted in high fatty acid consumption and marked fat loss, presumably through the disruption of glucose metabolism in skeletal muscles. To test this hypothesis, we generated conditional knockouts of *Tdp-43* using a muscle specific (MLC) driver of Cre recombinase. Loss of *Tdp-43* in skeletal muscle led to adult onset weight loss beginning about 3 months of age and premature death within a month. Pathological examination of skeletal muscles revealed degeneration of fibers characterized by the presence of vacuoles and inclusions, as well as marked regeneration, observations that are consistent with a myopathy occurring in these mice. Moreover, we observed significant reduction in levels of *Tbc1d1* and altered splicing of sortilin in skeletal muscle of these knockout mice. We hypothesize that loss of *Tdp-43* in skeletal muscle leads to disruption of glucose metabolism and preferential utilization of lipid fuel. We propose that this metabolic adaptation occurs as a result of impaired translocation of glucose transporter GLUT4 to the cell surface in a pathway that is regulated by GTPase activating protein *Tbc1d1* and Sortilin. Taken together, these findings suggest an important role for *Tdp-43* in skeletal muscle, and implicate defective energy metabolism in the progression of ALS and possibly a select group of myopathies.