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Hypermetabolism as a Risk Factor for ALS

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14. ABSTRACT 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.					
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Introduction

One of key Statement of work (SOW) in our proposal was to generate *MLC-Cre;TardbpF/F* mice to examine the role of Tdp-43 in skeletal muscle (see Task 2). Although TDP-43, an essential RNA binding protein initially linked to ALS-FTD¹, TDP-43 proteinopathy has also been associated with several degenerative diseases including various forms of Inclusion Body Myopathy^{2, 3}. We now observe that deletion of Tdp-43 from skeletal muscle leads to an IBM like mouse model which we now document in our report below. Recently, TDP-43 positive inclusions have also been described within muscle fibers of patients with various forms of myopathies characterized by rimmed vacuoles and inclusion bodies^{4, 5}. These include myopathies due to Valosin Containing Protein (VCP) mutations such as the multisystem disorder Inclusion Body Myopathy with Paget's disease of bone and Frontotemporal Dementia (IBMPFD), as well as non-VCP linked disorders like hereditary Inclusion Body Myopathy (hIBM) and sporadic Inclusion Body Myositis (sIBM). These disorders are characterized clinically by progressive muscle degeneration and weakness that can progress to marked disability. Our current understanding of mechanisms underlying the development and progression of muscle atrophy in IBM is limited. Defective autophagy/mitophagy is a pathologic feature of IBM³, however, it is unclear how TDP proteinopathy is mechanistically related to disruptions of these systems. The observation that nuclear clearance of TDP-43, accompanied by its accumulation in the sarcoplasm, is a hallmark in affected myofibers of cases of IBM suggests that chronic depletion of TDP-43 could contribute to the degeneration of skeletal muscle. Importantly, we show now that depletion of Tdp-43 in skeletal muscle recapitulate many aspects of human disease.

While we were performing these studies, we were also interested in whether depletion of Tdp-43 in motor neurons could also cause motor neuron disease in mice since we hypothesize that loss of Tdp-43 function in humans could lead to ALS. Thus, we now document below that deletion of Tdp-43 in motor neurons causes robust motor neuron disease. Because of direct relevance of this study to ALS, we have not focused on Task 1 proposed in our original SOW. In summary, we have now generated two relevant mouse model of human diseases, namely ALS and IBM, through support of this grant. Summarized below are the key data supporting these mouse models of human IBM and ALS.

Body

1. Reduction of Tdp-43 in skeletal muscle of *MLC-Cre;Tdp-43^{F/-}* mice. To test whether loss of TDP-43 function contribute to the pathogenesis of IBM, we generated mice lacking Tdp-43 in skeletal muscle termed, *MLC-Cre;Tdp-43^{F/-}*, from *Tdp-43^{loxP/loxP}*⁶, *Tdp-43^{+/-}* and *MLC-Cre⁷* mice. Expected Mendelian ratios were observed from such crosses, suggesting that Tdp-43 was not required for development of skeletal muscle during embryogenesis. Level of Tdp-43 was substantially reduced in skeletal muscle of *MLC-Cre;Tdp-43^{F/-}* mice as compared to that of *MLC-Cre;Tdp-43^{F/+}* or *Tdp-43^{F/+}* mice, but remained unaffected in cardiac muscle and brain. Immunohistochemical analysis of skeletal muscle with antisera directed against Tdp-43 corroborated with reduced accumulation of Tdp-43 in *MLC-Cre;Tdp-43^{F/-}* mice.

2. Loss of Tdp-43 in skeletal muscle leads to adult onset weight loss and premature death. Both *MLC-Cre;Tdp-43^{F/-}* and *MLC-Cre;Tdp-43^{F/+}* mice showed comparable growth and development into early adult. However, from 2.5 months in age for males and 3.5 months for females, *MLC-Cre;Tdp-43^{F/-}* mice exhibited a dramatic reduction in body weight and size. These mice reach end stage within 6 weeks after onset of weight loss. End stage in *MLC-Cre;Tdp-43^{F/-}* mice is characterized by an emaciated appearance, a pronounced hunch, inactivity and labored breathing. Gross dissection of mice lacking Tdp-43 at end stage show reduced muscle tone and

marked fat loss. Kaplan-Meier analysis of a cohort of mice indicates a median survival of 120 days for male and 140 days for female *MLC-Cre;Tdp-43^{F/-}* mice, while control littermates survived well past the span of the experiment. Consistent with reduced muscle tone, *MLC-Cre;Tdp-43^{F/-}* mice showed significant muscle weakness ($p=0.0013$) as measured by grip strength of both forelimbs and hindlimbs.

3. Mice lacking Tdp-43 in skeletal muscle develops a myopathy reminiscent of IBM.

Pathological analyses of 3 month old *MLC-Cre;Tdp-43^{F/-}* mice showed striking skeletal muscle atrophy, with little evidence of histological abnormalities in other organs, including the heart (cardiac muscle) and gastrointestinal tract (smooth muscle). Hematoxylin and Eosin (H&E) staining of skeletal muscle from *MLC-Cre;Tdp-43^{F/-}* mice showed fibers with hypercellularity, large variability in size and increased numbers of atrophic myofibers. There was marked regeneration as indicated by the large number of atypical, enlarged and pale staining nuclei in fibers; as well as scattered degeneration as evidenced by fibers with loss of striation, hyper-eosinophilic and rounded. Muscle samples taken from the quadriceps of *MLC-Cre;Tdp-43^{F/-}* mice at end stage (4.5 month old) show similar but more severe pathology to that observed in the 3-month-old mice. Skeletal muscle of the paraspine, cheek and tongue showed similar characteristics of degeneration and aberrant regeneration. Analyses of mice at 1.5 months showed mostly healthy developing muscle fibers. Taken together, pathological findings from skeletal muscles of *MLC-Cre;Tdp-43^{F/-}* mice indicate that loss of Tdp-43 from skeletal muscle is sufficient to induce a myopathy. Gomori Trichrome staining revealed striking numbers of red-rimmed vacuoles in *MLC-Cre;Tdp-43^{F/-}* mice, and these are a defining feature of IBM. Acid Phosphatase staining was increased in atrophic fibers of *MLC-Cre;Tdp-43^{F/-}* mice, reflecting increased lysosomal activity. Staining with NADH showed altered patterning of Type-I and Type-II fibers in *MLC-Cre;Tdp-43^{F/-}* mice, and several abnormal darkly stained fibers were present. The internal architecture was coarser in mice lacking Tdp-43, reflecting myofibrillar disruption and aberrant immature regenerating fibers. There were also areas with irregular deposits of stain that would indicate uneven distribution of mitochondria. Cytochrome C Oxidase/Succinate Dehydrogenase (COX/SDH) staining of muscle sections confirms aberrant mitochondrial activity and abnormal mitochondrial accumulation in *MLC-Cre;Tdp-43^{F/-}* mice. Collectively, immunohistological data show pathological features that are characteristic of IBM, and strongly support the idea that the myopathy observed in *MLC-Cre;Tdp-43^{F/-}* mice resembles that of IBM.

4. Massive degeneration of mitochondria in *MLC-Cre;Tdp-43^{F/-}* mice.

Finally, ultrastructural analyses of quadriceps and gastrocnemius muscles demonstrated substantial pathology in *MLC-Cre;Tdp-43^{F/-}* mice compared to controls. In control *Tdp-43^{F/+}* mice, myocytes show regular banding pattern with morphologically normal mitochondria located adjacent to the Z disks; and each myocyte had a peripherally located nucleus. In contrast, mice lacking Tdp-43 in skeletal muscle had large numbers of myocytes with disintegrated myofilament and one or more centrally located nuclei. There was marked redistribution of mitochondria into aggregates which tended to accumulate around amorphous deposits, and many of the mitochondria were giant and swollen with degenerate cristae. The presence of a large number of abnormal mitochondria is suggestive of a defect in mitophagy - the selective autophagy of damaged mitochondria. Vacuoles can be seen in some fibers, although it is unclear whether these are lysosomal in nature or reflect swollen mitochondria with degenerate cristae. Another prominent feature seen clearly in the longitudinal sections is the presence of membranous whorls of autophagosomal/lysosomal debris within degenerate myofibers. Importantly, the abnormal accumulation and degeneration of mitochondria observed in *MLC-Cre;Tdp-43^{F/-}* mice have also been reported in IBM muscle biopsies⁸.

5. Pathways impacted by Tdp-43 deficiency in skeletal muscle. To determine the mechanism by which loss of TDP-43 in skeletal muscle leads to myofiber degeneration, we assessed the impact of loss of Tdp-43 on the transcriptome of skeletal muscle from *MLC-Cre;Tdp-43^{F/-}* mice. RNA was isolated from the quadriceps of *MLC-Cre;Tdp-43^{F/-}* and control *Tdp-43^{F/+}* mice at 2 months, at which time there was little or no pathologic alterations seen with H&E and Gomori Trichrome stains but COX-deficient fibers were present. Expression profiling and functional analyses identified changes in expression levels and alternatively spliced variants of a large number of transcripts that may contribute to the myopathy seen at end stage. Whereas a large number of genes were significantly downregulated, a smaller but substantial number of transcripts were upregulated in skeletal muscle of *MLC-Cre;Tdp-43^{F/-}* mice. A significant proportion of the transcripts that were downregulated encodes mitochondrial proteins and processes, which would suggest aberrant mitochondria as a cause for the myopathy observed at end stage of *MLC-Cre;Tdp-43^{F/-}* mice. Transcripts encoding proteins involved in the lysosomal degradation pathway and apoptotic genes were among those that were significantly upregulated. Several transcripts related to regeneration of skeletal muscle and embryonic forms of some developmental genes were also increased. These findings suggest multiple pathways could be impacted by TDP-43 in skeletal muscle.

6. Deletion of Tdp-43 in motor neurons is sufficient to cause motor neuron disease.

To test whether depletion of Tdp-43 in neurons is sufficient to cause age-dependent neurodegeneration, we crossbred our conditional *Tardbp* knockout mice⁶ with *ChAT-IRES-Cre* mice to generate mice lacking *Tdp-43* in spinal motor neurons (*ChAT-IRES-Cre;Tardbp^{F/F}* mice). To determine the rate of Cre-dependent excision, we assessed the levels of Tdp-43 in spinal cords of *ChAT-IRES-Cre;Tardbp^{F/F}* mice. Cre-dependent excision was highly efficient in *ChAT-IRES-Cre;Tardbp^{F/F}* mice, as essentially all ChAT positive neurons in ventral horn of the spinal cord failed to accumulate Tdp-43. To assess the consequences of loss of Tdp-43 function in motor neurons of spinal cord, *ChAT-IRES-Cre;Tardbp^{F/F}* mice showed reduced body weight as compared to that of control littermates and failed to grow after 13 week of age. Moreover, *ChAT-IRES-Cre;Tardbp^{F/F}* mice exhibited tremors, abnormal claspings, abnormal gait and weakness. Significantly, hindlimbs of *ChAT-IRES-Cre;Tardbp^{F/F}* mice were paralyzed ~7 months of age and reached end-stage (as defined by failure to right themselves within 10 seconds when placed on their back) between 7-8 months of age. These phenotypes observed in *ChAT-IRES-Cre;Tardbp^{F/F}* mice are consistent with motor neuron disease and support the view that loss of TDP-43 in motor neurons is a major contributing factor in the pathogenesis of ALS-FTD.

Key Research Accomplishments

- To address the role of Tdp-43 in skeletal muscle, we generated a Tdp-43 mouse model that lack Tdp-43 specifically in skeletal muscle.
- Diminished amount of Tdp-43 in skeletal muscle led to adult onset weight loss beginning at ~3 months of age and 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.
- Immunohistochemical and ultrastructural analysis of mice lacking Tdp-43 in skeletal muscle showed pathological features that are characteristic of IBM.
- RNA-seq and bioinformatic analysis revealed some important pathways impacted by Tdp-43 in skeletal muscle.
- These results indicate that we have generated a mouse model of IBM.
- Our findings support the idea that Tdp-43 plays an important physiological role in skeletal muscle and implicate the participation of Tdp-43 in the pathogenesis of IBM.
- To address the role of Tdp-43 in motor neurons, we generated a Tdp-43 mouse model that lack Tdp-43 specifically in lower motor neurons.

- Analysis of mice lacking Tdp-43 in motor neurons showed evidence of age-dependent motor neuron disease.
- Our findings support the idea that loss of Tdp-43 function plays an important role in motor neurons and implicate depletion of Tdp-43 as a key pathogenic mechanism in ALS.

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). Importantly, we are writing manuscripts to document these novel findings and will be submitting this work for consideration of publication in the near future.

Conclusion

Our work has uncovered major pathological hallmarks of IBM in the *MLC-Cre;Tdp-43F/-* mouse, thus making this mouse as an attractive model for testing disease mechanisms contributing to muscle degeneration in IBM. We believe this mouse model of myopathy will be instrumental in unraveling the pathogenic mechanisms underlying diseases such as IBM or IBMPFD in the future. Moreover, we have generated a valuable robust mouse model lacking Tdp-43 in motor neurons as a useful model system for testing mechanism and therapeutic strategy for ALS.

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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 CreloxP 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.

Supporting Figure Legends and Figures

Figure Legends

Fig. 1(a-b) Levels of Tdp-43 was substantially reduced in skeletal muscle of *MLCCre; Tdp-43F/-* mice (K) compared to *Tdp-43F/+* control mice (C). (c) *MLC-Cre; Tdp-43F/-* (K) demonstrated a significant decrease in weight from about 2.5 months for males and 3.5 months for females compared to *Tdp-43F/+* mice (C). (d) Median survival of *MLCCre; Tdp-43F/-* mice (K) was 120 days for males and 140 days for females, control littermates (C) survived well past the span of the experiment ($p < 0.0001$). (e) *MLCCre; Tdp-43F/-* mice $n=6$ showed significant reduction in muscle strength ($p=0.0013$) compared to control littermates $n=8$. Unless indicated, all control groups refer to *MLCCre; Tdp-43F/+*, *Tdp-43F/+* and *Tdp-43F/-* littermates.

Fig. 2(a-e) Immunohistochemical stainings of quadriceps show pathological changes in *MLC-Cre; Tdp-43F/-* mice (right panels) compared to control *Tdp-43F/+* mice (left panels). These changes have features that resemble that of Inclusion Body Myopathy (refer to text for detailed description). Error bars represent $50\mu\text{m}$.

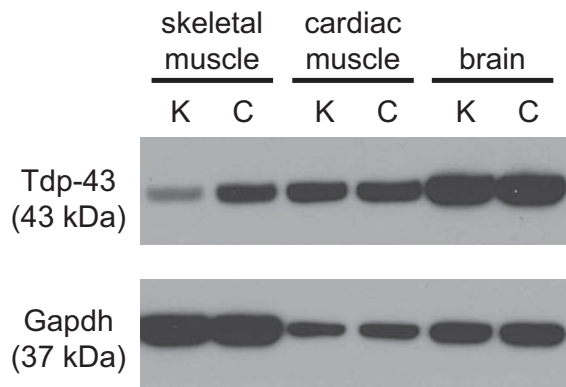
Fig 2(a) H&E shows myofiber atrophy (denoted by arrowhead), as well as marked increase in numbers of internal nuclei (*) that are enlarged and pale staining. (b) Gomori Trichrome reveals striking numbers of red-rimmed vacuoles (*) that is a defining feature of Inclusion Body Myopathy. (c) Increased Acid Phosphatase staining (*) suggests increased lysosomal activity, (d) Uneven NADH staining reflects disruption of myofibrillar architecture (*) and abnormal accumulation of mitochondria (arrowhead). (e) Cytochrome C Oxidase (COX) stains brown and Succinate Dehydrogenase (SDH) stains blue. There is marked increase in COX-deficient and SDH-dark fibers, findings typical of mitochondrial pathology.

Fig. 3(a) There is a marked decrease in Atg-7 that is accompanied by an increase in the ratio of LC3-I to LC3-II in skeletal muscle extracts of 2 month old and 4.5 month old *MLC-Cre; Tdp-43F/-* mice compared to control *Tdp-43F/+*. (b) Accumulation of p62+ ubiquitin+ inclusions in the sarcoplasm of *MLC-Cre; Tdp-43F/-* but not *Tdp-43F/+* mice. (c) Myocytes of *Tdp-43F/+* mice have regular banding pattern with peripherally located nuclei (*) and morphologically normal mitochondria located adjacent to Z-disks. (d) Myocytes of *MLC-Cre; Tdp-43F/-* mice have disintegrated myofilament (loss of banding pattern) with amorphous sarcoplasmic deposits (#), redistribution of mitochondria (*) and aberrant nuclei (denoted by arrow). (e) Accumulation of swollen degenerate mitochondria (*) around sarcoplasmic inclusions (#) were common. (f) Accumulation of vacuole-like structures (arrowhead) within myofibers of *MLC-Cre; Tdp-43F/-* mice. (h) Membranous whorls of autosomal/lysosomal debris within degenerate myofibers in *MLC-Cre; Tdp-43F/-* muscle. Error bars represent $50\mu\text{m}$ (b), $10\mu\text{m}$ (c-d) and 500nm (e-g).

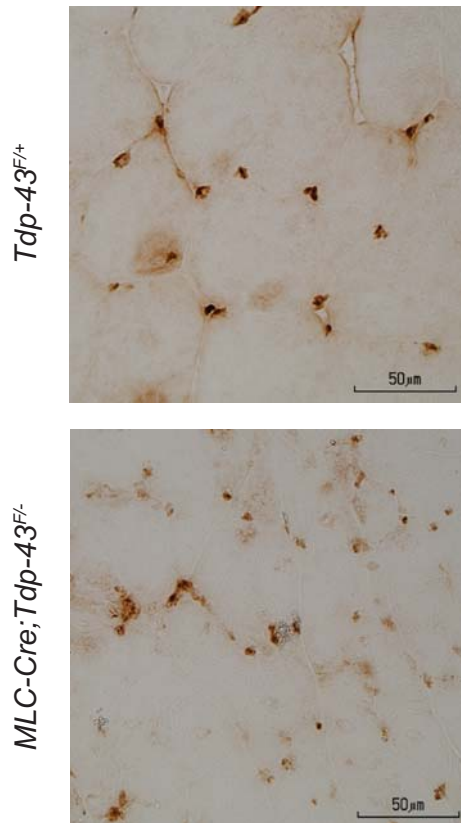
Fig. 4 (a) Breeding strategy to generate *ChAT-IRES-Cre; Tardbp^{F/F}* mice. (b) Reduction of Tdp-43 levels in *ChAT-IRES-Cre; Tardbp^{F/F}* mice. Spinal cord lysates from 1, 2, 4, and 7 month old *ChAT-IRES-Cre; Tardbp^{+/+}*, *ChAT-IRES-Cre; Tardbp^{F/+}* and *ChAT-IRES-Cre; Tardbp^{F/F}* mice were immunoblotted with antibody against Tdp-43. GAPDH antibody was used as a loading control. (c) Immunofluorescence staining of ventral spinal cord of p21 *ChAT-IRES-Cre; Tardbp^{+/+}* and *ChAT-IRES-Cre; Tardbp^{F/F}* mice with antisera to ChAT (green) and TDP-43 (red); nuclei stained with DAPI (blue). Scale bar, $50\mu\text{m}$. Note the depletion of nuclear Tdp-43 staining in neurons of CA3 and motor neurons. (d) Reduction in body weight of *ChAT-IRES-Cre; Tardbp^{F/F}* mice. Error bars indicates SEM.

Figure 1

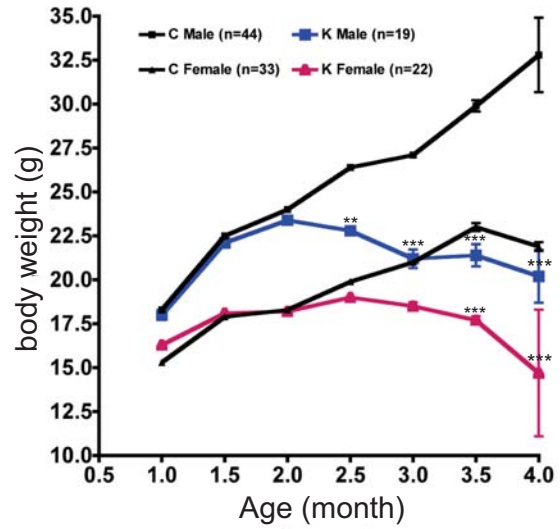
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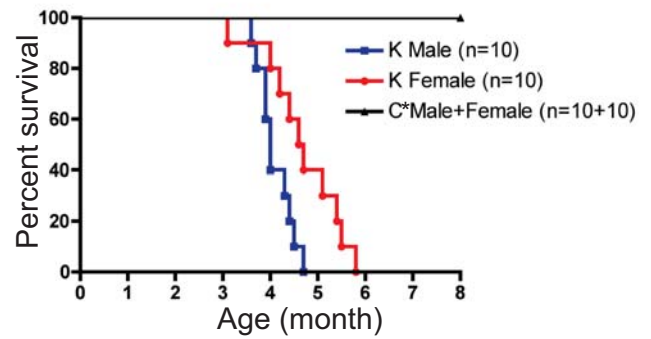
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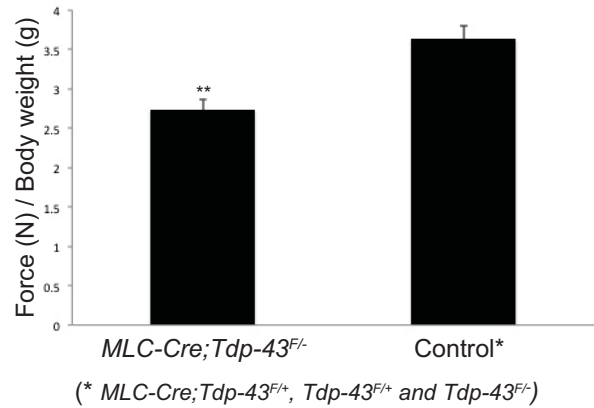


Figure 2

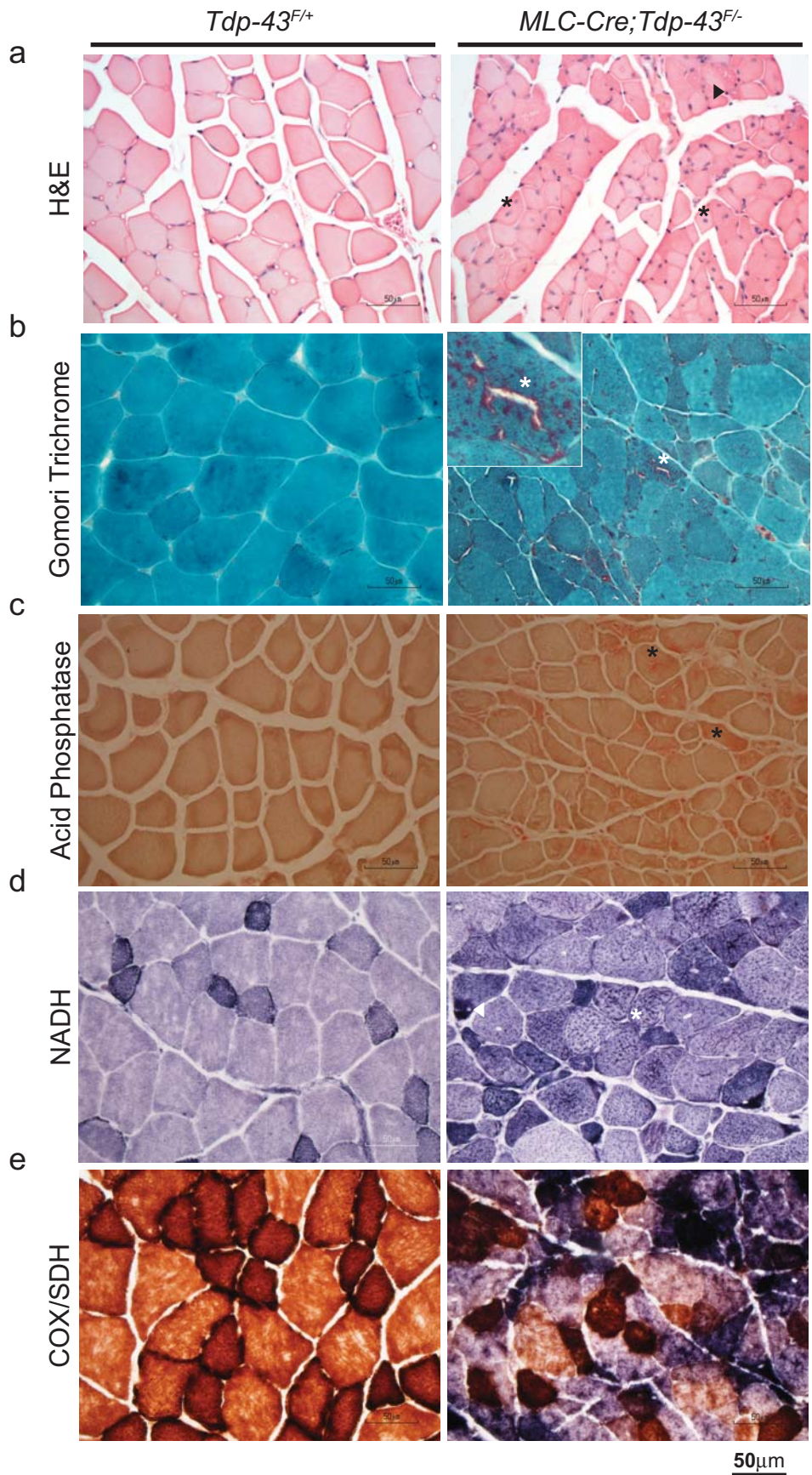
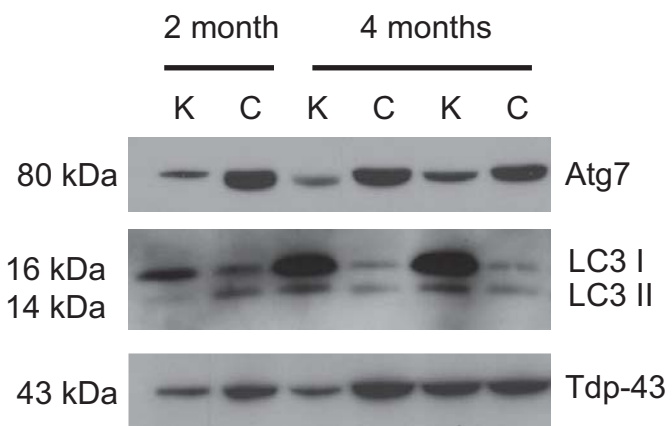
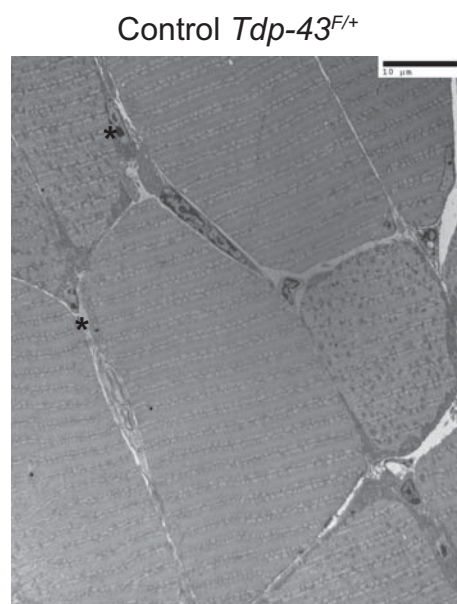


Figure 3

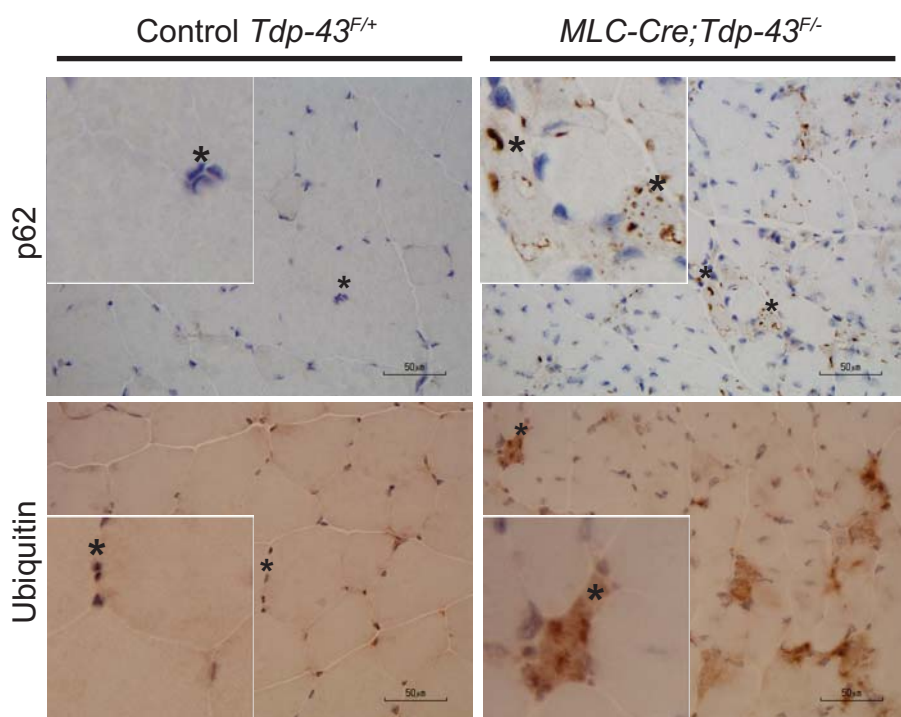
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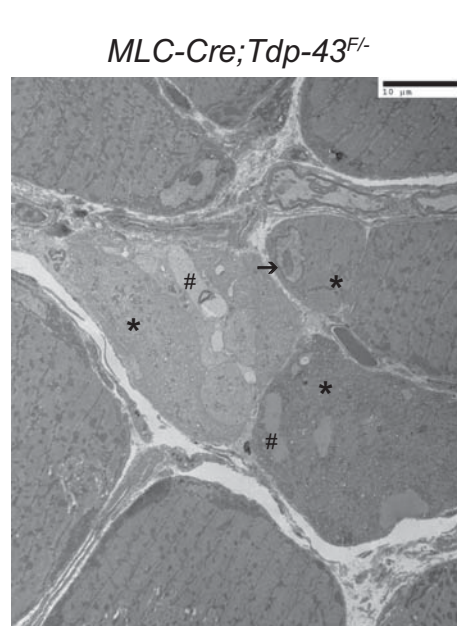
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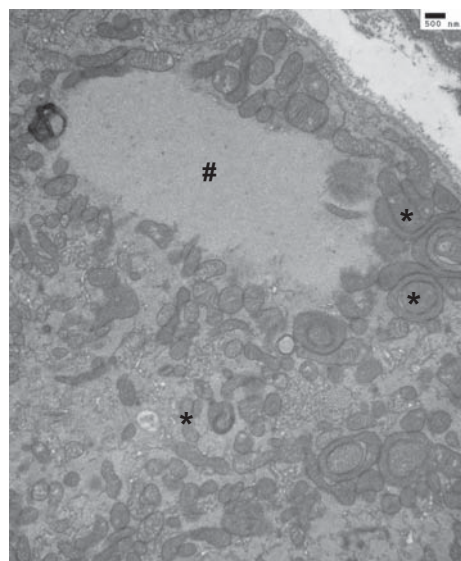
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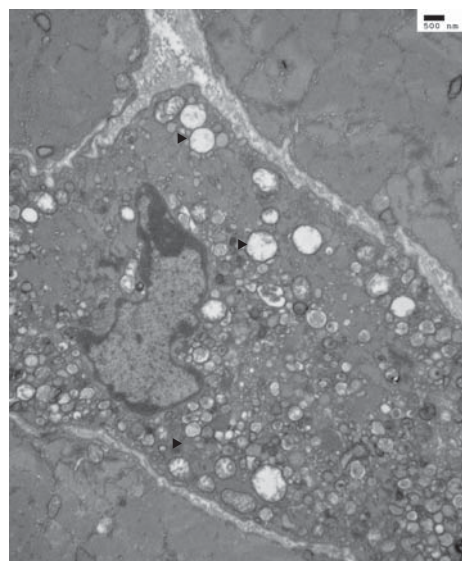
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