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TITLE: Suppression of TDP-43 Proteinopathy in Mice by Targeting Rad-23

PRINCIPAL INVESTIGATOR: Robert Kalb

CONTRACTING ORGANIZATION: Northwestern University, Chicago, IL

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Suppression of TDP-43 Proteinopathy in Mice by Targeting Rad-23

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6. AUTHOR(S)
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5e. TASK NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Protein homeostasis (or "proteostasis") is the balance of protein synthesis and protein degradation and is a requirement for optimally cellular functioning. The observation that misfolded proteins aggregate and accumulate in all neurodegenerative diseases is prime evidence that proteostasis has gone awry. Re-establishment of proteostasis is a major therapeutic goal. The identification of misfolded proteins and their targeting to the major degradative pathways (i.e., the proteasome or the autophagy pathway) are highly regulated processes. Rad23 was originally identified in a yeast screen for mutants that were hypersensitive to UV irradiation and subsequent shown to play a critical role in the ubiquitin- proteasome system for protein degradation. Ablation of rad23 accelerates the destruction several disease-causing, aggregation-prone mutated proteins and confers benefits in a variety of model systems. Mammals have 2 rad23 isoforms, rad23A and rad23B. The objective of this proposal is to use genetic approaches and antisense oligonucleotide (ASO) technology to reduce the abundance of rad23 in mouse models of Amyotrophic Lateral Sclerosis, ALS. We will use the Thy1-TDP-43 transgenic mouse (TAR4/4) created by the lab of Samir Kumar-Singh. We hypothesize that loss of rad23 will accelerate the destruction of misfolded TDP43, promote proteostasis and delay or arrest disease progression.

15. SUBJECT TERMS

None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	12	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

In this project we study a mouse model of ALS in which wild type human TDP43 is overexpressed (OE) in the nervous system. Our goal is to determine if reducing the abundance of two proteins, RAD23A and/or RAD23B ameliorates the ALS phenotypes in the TDP43 OE mice. Two tools are brought to bear on the scientific question: 1) a mouse that is null for *rad23A*, and 2) antisense oligonucleotides (ASO) that target *rad23A* or *rad23B* when delivered intracerebroventricularly.

2. KEYWORDS:

ubiquitin-proteasomal system, TDP43, ASO, RAD23, Tar4/Tar4 mouse

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major tasks that support the specific aims and milestones

1. Determine if genetic ablation of *rad23A* ameliorates the TDP43 proteinopathy phenotypes exhibited by the TAR4/4 mouse (denoted below as "Relevant to Major Task #1")
2. Determine potency of ASOs to rad23A and rad23B in vivo. Subtask (a) – ICV administration of various concentrations of ASOs to mice. Subtask (b) – harvest brain and spinal cord for qPCR and western blot. Subtask (c) – determine time course of target knockdown after single administration of ASO. Subtask (d) – interrogate brain and spinal cord biochemically and histologically looking for evidence for toxicity. Subtask (e) – determine if ASO to rad23 evoke changes in autophagy using histological and biochemical tools. (denoted below as "Relevant to Major Task #2")
3. Deliver ASOs to rad23A and rad23B to the TAR4/4 mouse. Subtask (a) ICV administration of ASOs to P1-2 TAR4/4 mice. Subtask (b) – harvest brain and spinal cord for qPCR and western blot for rad23A, rad23B and mutant TDP43 (soluble, insoluble and misfolded). Subtask (c) – monitor disease progression in TAR4/4 mice, general. Record weight, activity, metabolic parameters, disease onset, duration and time to death. Subtask (d) – monitor disease progression in TAR4/4 mice, motor. Undertake strength testing on grip strength meter, rotarod endurance. Subtask (e) - monitor disease progression in TAR4/4 mice, histology. Undertake motor root axon counts, determination of astrocytosis, microgliosis, nmj intergrity. (denoted below as "Relevant to Major Task #3")

MAJOR ACTIVITIES.

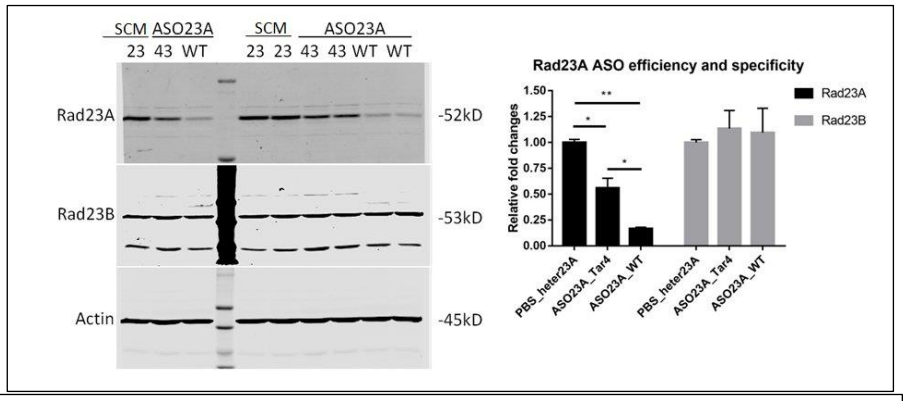
- Breeding mice to maintain colony and generate genetic doubles.
- Performed ICV injections of ASOs.
- Monitored gene KD.
- Monitored effect on survival of the ALS model mouse called Tar4/Tar4

SPECIFIC OBJECTIVES

- General a colony of mice with an appropriate background genetic strain that generates animals of interest (genotype Tar4/Tar4) on a weekly basis.
- Initiate creation of the genetic double, Tar4/Tar4;*rad23a*$^{-/-}$
- Master ICV injections to ensure high survival rates of postnatal day zero injected mice.
- Determine dose of ASO that does not have toxicity and leads to selective KD of target gene
- KD *rad23a* and/or *rad23b* in the Tar4/Tar4 mice; monitor effects on survival.

SIGNIFICANT RESULTS OR KEY OUTCOMES, INCLUDING MAJOR FINDINGS, DEVELOPMENTS, OR CONCLUSIONS (BOTH POSITIVE AND NEGATIVE)

- We have successfully established a large breeding colony of Tar4 mice with background genotype of B6SJL. "Relevant to Major Task #2 and #3"
- We have created the parental generation required to generate mice with genotype of Tar4/Tar4;*rad23a*$^{-/-}$ "Relevant to Major Task #1"
- We have established protocols and processes for ICV injection of ASO (both to *rad23* targets and scramble sequence controls) with a survival rate of injected P0 mice of greater than 95%. "Relevant to Major Task #2"
- We have established concentrations of ASO that target *rad23A* that lead to 82 % knockdown of RAD23A with no effect on the abundance of RAD23B. We have established concentrations of ASO that target *rad23B* that lead to 71 % knockdown of RAD23B with no effect on the abundance of RAD23A. "Relevant to Major Task #2"



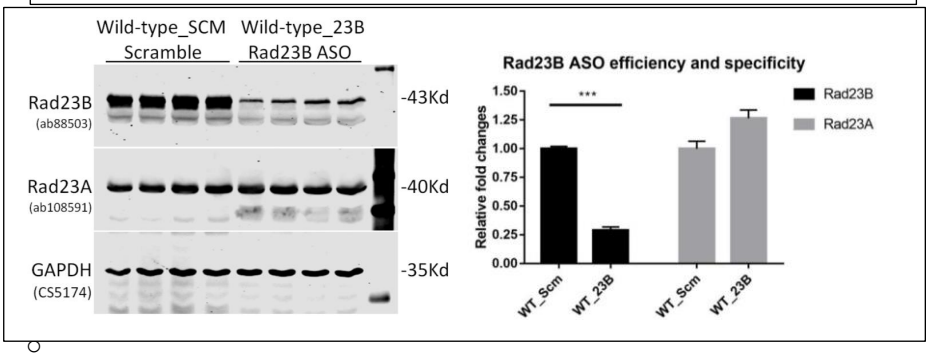
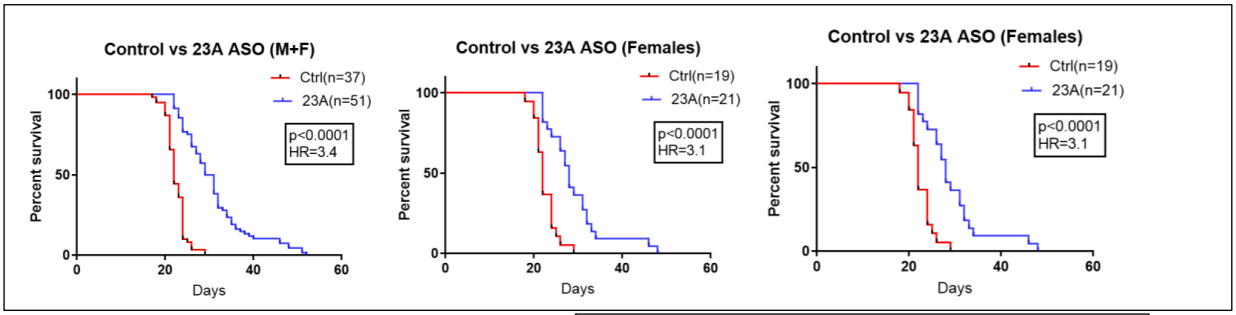


Figure legend: Upper text box. Western blot of brain lysates from WT animals and Tar4/Tar4 mice that received an intracerebroventricular (ICV) injection of scramble (SCM) sequence antisense oligonucleotide ASO or ASO targeting *rad23a* (ASO23A). ASO23A reduces the abundance of RAD23A in both wild type "WT", *rad23a*$^{+/-}$ heterozygous mice ("23") and Tar4/Tar4 ("43") animals but has no effect on the abundance of RAD23B or actin. Quantification of changes in western blot signal is see in bar graph to right (*: $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$). Lower text box. Western blot of brain lysates from WT animals that received an ICV injection of scramble SCM ASO or ASO targeting *rad23b* (ASO23B). ASO23B reduces the abundance of RAD23B in WT animals but has no effect on the abundance of RAD23A or actin. Quantification of changes in western blot signal is see in bar graph to right.

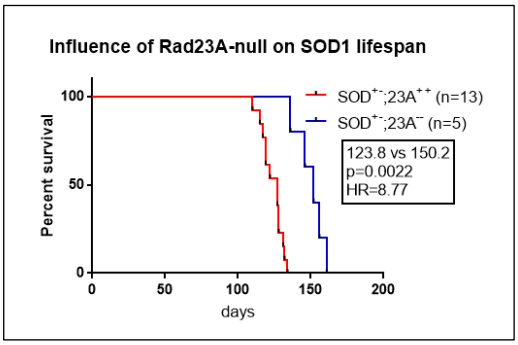
- We have successfully KD RAD23A in a sufficient number of Tar4/Tar4 to undertake sufficiently powered survival curves of males and females (compared with scramble sequence ASO injected animals). See top of next page. We have unambiguous evidence that reason targeting *rad23a* prolongs the life of both male and female Tar4/Tar4 animals (Relevant to Major Task #3)



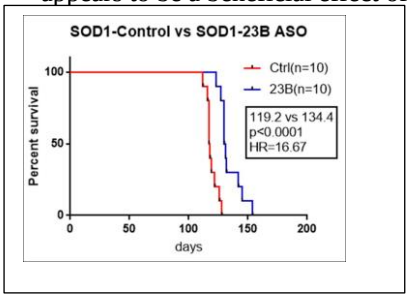
Male:
Gehan-Breslow-Wilcoxon test p value < 0.0001;
Hazard Ratio (logrank) (HR) is 4.063; Hazard Ratio (Mantel-Haenszel) (HR) is 19.12
Median survival days for control is 22 and for 23A is 31.5

Female:
Gehan-Breslow-Wilcoxon test p value < 0.0001;
Hazard Ratio (logrank) (HR) is 3.063; Hazard Ratio (Mantel-Haenszel) (HR) is 7.117
Median survival days for control is 22 and for 23A is 28

- We have generated 10 Tar4/Tar4;*rad23a*$^{-/-}$ and we plan to generate more animals with this genotype over time. At present the number of animals is too small to make any comments on survival or other parameters. "Relevant to Major Task #1"
- We have generated 13 G93A SOD;*rad23a*$^{-/-}$ animals. We do not have sufficient number to break this down by sex. Preliminarily the average survival of the G93A SOD mouse is 115-120 days and in the *rad23a*$^{-/-}$ background the G93A SOD mice live ~ 140 days. We need to generate more mice of the appropriate genotypes to make more firm statements. "Relevant to Major Task #1"



- We have generated <20 Tar4/Tar4 animals with ASO mediated KD of *rad23b* and we plan to generate more animals with this genotype over time. There maybe a problem with inter litter comparisons and so at present the number of animals is too small to make any comments on survival or other parameters. "Relevant to Major Task #3"
- We have generated 10 G93A SOD animals with ASO mediated KD of *rad23b* and we plan to generate more animals with this genotype over time. While the number of animals is small there appears to be a beneficial effect of ASO targeting *rad23b*. "Relevant to Major Task #3"



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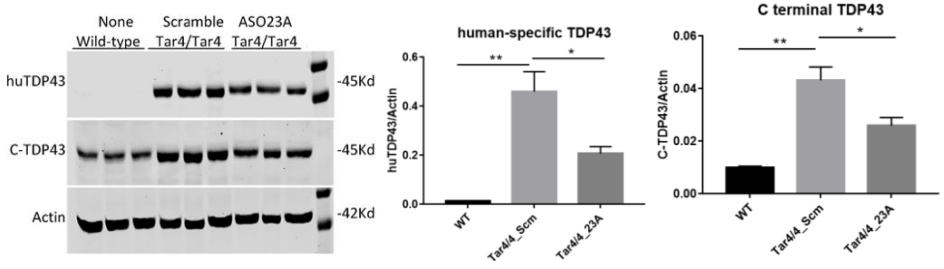
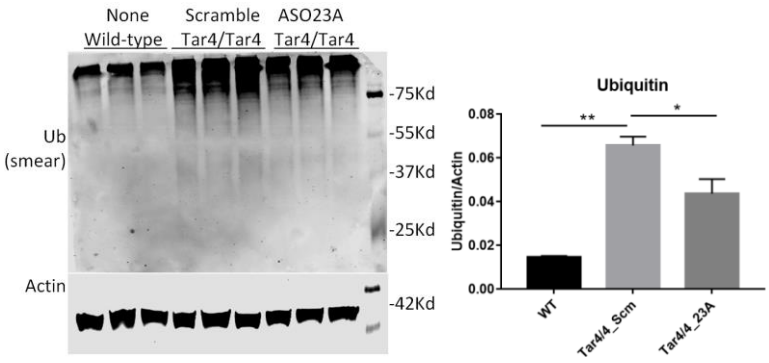
- We have performed biochemical interrogations of the brains from WT mice, Tar4/Tar4 scrambled ASO injected and Tar4/Tar4, *rad23a*-targeted ASO. Human TDP43 is seen in Tar4/Tar4 animals and the abundance of this species is reduced in animals treated with the *rad23a*-targeted ASO in comparison with the scrambled ASO. Human + mouse TDP43 was studied is seen in Tar4/Tar4 animals and the abundance of these species is reduced in animals treated with the *rad23a*-targeted ASO in comparison with the scrambled ASO. "Relevant to Major Task #2"



Effects of Rad23A ASO on levels of TDP43 in different forms. Mice received an intraventricular injection of ASO targeting rad23a or a scrambled sequence at P0 (n=3 per group). Cortex tissues were harvested at day 21 for western blot. Human TDP43 levels were significantly increased in Tar4/4 mice. Administration of Rad23A ASO reduced human TDP43 (huTDP43)l evels by 55%. An antibody that sees an epitope that is common between human and mouse TDP43 (C-TDP43) revealed a 44% reduction of TDP43. *: p<0.05; **: p<0.01 ; **: p<0.01. Bar graph quantification of data to right (*, p < 0.05; **, p < 0.01)

A smear of total ubiquitinated material is present in Tar4/Tar4 animals that is absent in the WT animals. The smear of total ubiquitinated proteins is reduced in animals treated with the *rad23a*-targeted ASO in comparison with the scrambled ASO. Quantification of these data is presented in bar graphs to the right "Relevant to Major Task #2"



Effects of Rad23A ASO on cellular whole ubiquitylation status. Mice were administrated at P0 with Rad23 A ASOs or scramble controls. Cortex tissues were harvested at d21 for western blot. TDP43 overexpression caused significant upregulation of ubiquitin in Tar4/4 mice. Administration of Rad23A ASO reduced TDP43-induced Ubiquitin levels by 36.6% . *: p<0.05; **: p<0.01.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report – plan to prepare manuscripts for publication after work is complete.

What do you plan to do during the next reporting period to accomplish the goals?

We need to complete the genetic double experiments and ICV injection of ASO to *rad23b* into the Tar4/Tar4 animals. Survival, biochemistry and histology will be performed.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

If we can show that KD of *rad23a* and or *rad23b* confer a benefit to mouse models of ALS, it is an important step towards motivating the Pharmaceutical Industry to target these genes for ALS therapeutics.

What was the impact on other disciplines?

Nothing to report at this time.

What was the impact on technology transfer?

Nothing to report at this time

What was the impact on society beyond science and technology?

Nothing to report at this time

5. CHANGES/PROBLEMS:

Nothing to report at this time

Actual or anticipated problems or delays and actions or plans to resolve them

None

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

Nothing to report at this time

Significant changes in use of biohazards and/or select agents

Nothing to report at this time

PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

  Not yet

  Books or other non-periodical, one-time publications.

  None

  Other publications, conference papers and presentations.

  None

- **Website(s) or other Internet site(s)**

  None

- **Technologies or techniques**

None

Inventions, patent applications, and/or licenses

None

Other Products

None

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: **Robert Kalb (No change)**

Name: **Xueshui Guo (No Change)**
Project Role: Postdoctoral Fellow
Nearest person month worked: 12
Contribution to Project: Dr. Guo has performed work in the area of

Name: **Danielle Cantoni**
Project Role: Research Technician
Nearest person month worked: 7
Contribution to Project: Ms. Cantoni has performed work in the area of mouse colony maintenance

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Change in Other Support is placed in bold.

ACTIVE

**Title: Suppression of TDP-43 Proteinopathy in Mice by targeting Rad-23*

Major Goals: To use genetic approaches and antisense oligonucleotide (ASO) technology to reduce the abundance of *wild type* rad23 (e.g, the TAR4/4 by the Kumar-Singh lab) in mouse models of Amyotrophic Lateral Sclerosis, ALS.
*Status of Support: Active
Project Number: W81XWH-21-1-0236
Name of PD/PI: Kalb, Robert
*Source of Support: Department of the Army
*Primary Place of Performance: Northwestern University Chicago
Project/Proposal Start and End Date: (MM/YYYY) (if available): 04/01/2021 – 03/31/2023
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	2.40
2. 2023	2.32

**Title: RAD23 control of ALS phenotypes*

Major Goals: To determine if mSOD or mTDP43 acts as an allosteric proteasome inhibitor in a rad23-dependent manner; determine if ablation of rad23A is beneficial in slow progressing *familial mutant* TDP43 mouse models (e.g., the bigenic rNLS8 mouse by Lee and Trojanowski Lab, the $TDP43^{Q331K}$ mouse, line 103 by Cleveland lab, and the Q331K knockin mouse by Sreedharan Lab); determine if knockdown of rad23B in the rad23A;slow progressing TDP43 mice is beneficial; determine if co-knockdown of rad23A and rad23 confers benefits in slow progressing TDP43 mouse models.
*Status of Support: Active
Project Number: RO1 NS122908
Name of PD/PI: Kalb, Robert
*Source of Support: NIH/NINDS
*Primary Place of Performance: Northwestern University Chicago
Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/01/2021-05/31/2026
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	3.33
2. 2023	4.20
3. 2024	4.20
4. 2025	4.20
5. 2026	4.20

****Title: Defining mechanisms underlying C9orf72-associated frontotemporal dementia with C. elegans and mammalian models***

Major Goals: Major Goals: In specific aim 1, we plan to determine if speckled-type BTB/POZ protein (SPOP) and diamino acid peptide repeat (DPR) proteins directly or indirectly interact to regulate DPR toxicity; in specific aim #2, we plan to determine how SPOP targets such as BRD proteins modulate DPR toxicity, and in specific aim #3 we will determine if SPOP inhibitors modulate DPR phenotypes in mammalian neurons and patient derived iPS cells differentiated into motor neurons.
*Status of Support: Active
Project Number: R01 NS124802
Name of PD/PI: Kalb, Robert
*Source of Support: NIH/NINDS
*Primary Place of Performance: Northwestern University Chicago
Project/Proposal Start and End Date: (MM/YYYY) (if available): 2/1/22 – 1/31/27
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	2.74
2. 2024	3.60
3. 2025	3.60
4. 2026	3.60
5. 2027	3.60

What other organizations were involved as partners?

Nothing to report

7. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: None

QUAD CHARTS: No

8. APPENDICES: No