AD_____

AWARD NUMBER: W81XWH-15-1-0569

TITLE: Development of a Novel Targeted RNAi Delivery Technology in Therapies for Metabolic Diseases

PRINCIPAL INVESTIGATOR: Michael P. Czech, Ph.D.

RECIPIENT: UNIVERSITY OF MASSACHUSETTS
WORCESTER MA 01655-0002

REPORT DATE: December 2018

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Dec 2018	FINAL	30 Sep 2015-29 Sep2018

4. TITLE AND SUBTITLE

Development of a Novel Targeted RNAi Delivery Technology in Therapies for Metabolic Diseases

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0569

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) **Michael P. Czech, PhD**

E-Mail:Michael.Czech@umassmed.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University Of Massachusetts Medical School 55 Lake Ave N Worcester MA 01655

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our objective is to develop a means of targeting siRNA molecules to relevant cells of the liver in order to silence genes that play key roles in inflammatory, metabolic or other pathways that cause nonalcoholic steatohepatitis (NASH) initiation or progression.. During this project we discovered that covalent coupling of a moiety (e.g., cholesterol or GalNAc) that promotes entry into cells directly onto the siRNA (forming "self-delivery "sd" RNAs) is a superb approach. During this past year we have focused on cholesterol covalently attached to the modified siRNA to determine efficiency for gene silencing in mice. Importantly, we have chemically modified the siRNA segment of the therapeutic to attenuate nuclease-mediated degradation, immune responses and toxicity and prolong gene silencing for weeks after one injection. We found both hepatocytes and Kupffer cells are well targeted by these formulations, with high efficiency and tissue specificity in gene silencing after single subcutaneous injections. We have identified superb target genes (TAZ and MCT1) for NASH, and high potency sdRNAs against these genes after exhaustive screening of sdRNA sequences. We have accomplished the silencing of TAZ selectively in liver with a potent Chol-sdRNA and a potent GalNAC-sdRNA, a major milestone. Importantly, we have now also reached our key milestone of the project, demonstrating a strong beneficial effect of Chol-sdRNA in a NASH mouse model.

15. SUBJECT TERMS

Fatty liver disease, steatohepatitis, RNA interference, Kupffer cells, hepatocytes, self delivery, sdRNA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	20	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

Liver steatosis and inflammation, resulting in the disease state denoted NASH, is often associated with obesity and type 2 diabetes, which have increased in incidence to nearly 10% of the US population. It is estimated that over the coming years, the consequences of NASH will be the major reason for liver transplants in this country. Type 2 diabetes is even more prevalent in our veterans, occurring in the range of 20%. Our project is designed to address this extreme medical need by designing a novel approach to inhibit fatty liver and decrease inflammation in the liver in obesity. Our approach is to use powerful siRNA-mediated gene silencing to decrease the expression of one or more genes that advance this disease state, thus alleviating the disease. By targeting and silencing such genes that promote the disease, we hypothesize that we can maintain a disease free state, and we are testing this hypothesis in mice. We are using what we denote "self-delivery (sd)" siRNA (denoted sdRNA) because a hydrophobic molecule (e.g., cholesterol) or a liver receptor ligand (e.g., GalNAc) attached to a stabilized siRNA (denoted Chol-sdRNA or GalNAc-sdRNA, respectively) becomes able to penetrate cells and silence genes in the absence of a potentially toxic transfectant-type agent.

The initial stages in our research have been directed to optimize an sdRNA formulation that is best able to silence genes in hepatocytes and other liver cell types, using a test gene as a target (gene PP1B) that we know siRNA can strongly silence. We accomplished this goal by subcutaneous injection of a potent Chol-sdRNA targeting PPIB and assessing its inhibited expression in liver. This past year our work has been aimed at identifying "disease" target genes, that when silenced, alleviate fatty liver and NASH, and developing potent Chol-sdRNAs that we predict will silence such genes. We have focused on the TAZ and MCT1 genes, which from the literature and our own work indicate that when effectively silenced, will alleviate NASH in mice. Most impactful has been our demonstrations that either a tool Chol-sdRNA or tool GalNAC-sdRNA we developed after much trial and error screening, have potent silencing effects on the TAZ target gene. **Finally, we have now demonstrated a beneficial effect of our tool Chol-sdRNA compound against TAZ in a mouse model of NASH, the key proposed milestone of the project.**

We are very excited about the progress of our work this past year, as the project really gained terrific traction and we can clearly see a track forward to bring this technology into the clinic over the next few years, assuming continued funding.

2. KEYWORDS:

Fatty liver disease, RNA interference, Kupffer cells, hepatocytes, siRNA, self-delivery siRNA

3. ACCOMPLISHMENTS of this project:

We have made rapid progress toward our goals, accomplishing the key milestone achievements by demonstrating that, 1.) our most potent Chol-sdRNA and GalNAC-sdRNA tool compounds that target the TAZ gene are able to silence this gene selectively in liver following a single subcutaneous injection in mice, and 2.) our Chol-sdRNA(TAZ) compound is able to alleviate NASH in a mouse model of this disease. This milestone achievement caps the culmination of many accomplishments along the way, **as summarized below:**

- **Accomplished:** We demonstrated that our Chol-sdRNA formulation directed against a test gene, PPIB, is consistently operational and working *in vivo,* following a single subcutaneous injection.
- **We accomplished** the strategy of identifying 3 metabolic genes that upon silencing are known to protect the liver against steatosis: genes MCT1, IHH and TAZ identified. These have become our focal points in developing a therapeutic sdRNA.
- **Accomplished:** Dozens of putative target sdRNA sequences were identified for synthesis in each of the three above genes using computer analysis of the entire gene sequences. All were synthesized as sdRNA strands, both sense and anti-sense.
- **Accomplished:** All the sdRNA strands that were synthesized for the TAZ gene were then manually annealed into double strand sdRNAs, providing us with 48 sdRNAs directed against TAZ to be tested for efficacy in *in vitro* assays. Thus, annealing all TAZ sdRNAs was accomplished.
- **We have now accomplished** the first phase of this strategy for the TAZ and MCT1 genes by generating the psiCHECK-2 vector with MCT1 and TAZ sequence targets inserted. Thus we generated both TAZ and MCT1 target genes in format for screening sdRNAs in cells *in vitro.* Similar experiments are in progress to accomplish this for the IHH gene.
- **Accomplished:** All the sdRNA strands that were synthesized for the TAZ and MCT1 genes were manually annealed into double strand sdRNAs, providing us with 48 sdRNAs directed against MCT1 and 48 against TAZ for testing for efficacy in *in vitro* assays.
- **Accomplished:** We employed our rapid throughput assay in our lab that allows us to test efficacy of siRNAs in cells in culture (denoted the Dual-Glo assay) for both TAZ and MCT1 genes. The screening of the 48 sdRNAs each against TAZ and MCT1 using this system produced numerous hits of high quality.
- **Accomplished:** Full dose response relationships were established for the top sdRNA hits for both TAZ and MCT1 gene targets. These data show high efficacy and potency, now allowing us to choose lead sdRNA compounds for testing against the endogenous genes *in vitro*. All this work was done with Cholesterol conjugated to the sdRNAs for delivery.
- **Accomplished:** Potent silencing of the **endogenous** NASH target gene TAZ was accomplished in cell culture *in vitro.* This showed that our screen of 48 Chol-sdRNAs yielded a potent compound that could target and silence the endogenous gene, not just the gene in an artificial vector.
- **Accomplished:** Each of the above itemized accomplishments for the Chol-sdRNA(TAZ) have now also been replicated and accomplished for GalNAC-sdRNA against TAZ, yielding a superb tool GalNAC-sdRNA compound for this target gene.
- **Accomplished:** Just over the past few weeks, we were able to demonstrate that our best tool compounds Chol-sdRNA and GalNAC-sdRNA against the TAZ gene effectively silenced TAZ in liver cells and selectively in hepatocytes, respectively, after a single subcutaneous injection. This is particularly important, **accomplishing a key milestone achievement of this project by demonstrating that our most potent Chol-sdRNA tool compound that targets the TAZ gene is able to silence this gene selectively in liver following a single subcutaneous injection in mice.**

- **Accomplished: <u>Achieved the proposed final milestone of the project</u> by demonstrating a beneficial effect of our Chol-sdRNA(TAZ) compound in a mouse model of NASH through bi-weekly subcutaneous injections.**

<u>Major goals and objectives:</u>

<u>Over the course of this 3 year project, we focused on meeting our primary goal of the original proposal, which was meeting the key milestone of testing a tool sdRNA compound in a NASH mouse model and achieve beneficial effects.</u>

The major objective in this project has been to develop and test effective candidate self delivery RNA formulations in diet-induced fatty liver disease models. Due to the advances noted in our previous reports, we showed that our new cholesterol-sdRNA formulations are more effective than the original proposed GeRPs and that our new disease gene targets TAZ and MCT1 are better than RIP140 to target in hepatocytes. The goal of alleviating NASH has remained the same, but the sdRNA formulations and target gene have been improved in the last year over the previous two years of the project.

We originally proposed that this final key goal of silencing one or more genes to alleviate hepatosteatosis in mouse models of obesity would be accomplished over this last year (MAJOR months 13-36 in the original SOW). Thus this Major Task 3 in Aim 2 has been our primary goal over the last year of the grant funding. As summarized above, we have demonstrated that our best tool Chol-sdRNA compound is effective *in vivo* at silencing TAZ—the data to support this key conclusion and milestone is shown below in the detailed report. We have therefore accomplished the first part of Major task 3, and we are excited to report we have also accomplished the second half of Major task 3, which is to show efficacy in the function of the TAZ-Chol-sdRNA. The latter achievement is demonstrated in preliminary experiments, with much more to do to replicate, solidify and expand this achievement. **We are hopeful to achieve funding in the EXPANSION award program to fully accomplish this key milestone in additional NASH models and confirm in an independent laboratory.** We are poised to execute the experiments to fully accomplish major task 3 in the DOD EXPANSION award and to move to an IND and the clinic.

<u>Detailed report of the Accomplishments (summarized above) under these goals:</u>

At the beginning of the year 2018, we were at the stage where we had progressed to successful gene silencing in liver with a test gene. Thus, a single subQ injection of Cholesterol (Chol)-self delivery (sd) RNA against the test gene PPIB was able to silence that gene in hepatocytes over a 2 week period, but not for 4 weeks (detailed in Annual Progress Report 2, October 29, 2017). This demonstrated that we had developed the technology to silence genes in liver using the Chol-sdRNA formulation with a single dose and single injection. These experiments reported in the annual progress report brought us to the stage where we could now test for the silencing of metabolic genes such as RIP140, which we believed would bring beneficial metabolic effects and advance the goal of alleviating NASH. However, we were surprised that we did not achieve this result when we did that experiment. As expected, the PPIB Chol-sdRNA had no effect on the RIP140 gene expression but did silence the PPIB gene, as it should. However, the RIP140 Chol-sdRNA did not cause either RIP140 silencing, as anticipated it would, nor PPIB silencing, as it shouldn't.

As previously conjectured, we believe the most likely reason that we did not observe knockdown with the RIP140 sdRNA in vivo in this experiment relates to the fact that even our best RIP140 Chol-sdRNA compound (3936) did not achieve the level of 75-80% knockdowns in cultured cells

that we were able to see with the positive control PPIB Chol-sdRNA. We have noticed in past experience that to obtain significant silencing *in vivo*, one must start with a "heroic" construct that shows extremely good silencing capability in cultured cells *in vitro*. It may be that the structure of RIP140 RNAs, at least all the Chol-sdRNAs that we screened so far, are simply not capable of providing this level of extreme silencing. It is possible that the structure of the sdRNA for the best RIP140 sequence is not in a 3D structure that optimizes silencing of the mRNA. This happens for some genes, and is not possible to predict from primary sequence data.

Based on this obstacle and rationale for the data obtained, we then decided to screen for super active Chol-sdRNA sequences against additional metabolic genes in the second quarter of project year 3 in order to be able to demonstrate silencing in vivo. Based on all the literature we reviewed and discussed internally, and all our previous experience with the biology of liver metabolism and NASH, we chose three top priority new genes that when silenced are strongly predicted to have a major beneficial effect on NASH. These predictions have very solid support from published data and our own experience—they are the TAZ, MCT1 and IHH genes.

Overview of the current strategy we pursued in this project:

1. Identify target sequences for sdRNAs in each of the three target genes TAZ, MCT1 and IHH that have characteristics amenable to being good RNAi targets.
2. Synthesize at least 48 sdRNA strands (both sense and antisense) against those target sequences in each of the three target genes, and anneal into double stranded sdRNAs.
3. Prepare and generate synthetic "target" cDNAs (denoted "target continuum") that have all the target sequences of a target gene in one cDNA so that it can be expressed in target cells in vitro and used as a surrogate target for high throughput screening in the Dual Glo assay.
4. Screen all the sdRNAs we have synthesized for each gene target (TAZ, MCT1 and IHH) against the specific "target continuum" for that gene using the Dual Glo assay.
5. Select hyper-active sdRNAs for each gene that are able to cause marked silencing *in vitro* and rescreen them in hepatocytes against the endogenous gene for silencing, then select sdRNAs that are able to silence at the level of at least 80% for *in vivo* testing.
6. Test the selected sdRNAs *in vivo* by single subcutaneous injection for effective silencing according to the protocols we developed for our test gene PPIB.
7. Once silencing is established *in vivo,* use the most potent Chol-sdRNA compounds against TAZ, MCT1 or IHH in NASH mouse models and determine efficacy on triglyceride accumulation, inflammation and fibrosis.

Pursuit of this Strategy:

Using both standard and unique principles of sequence selection that we have previously used in this project and reported, we screened the nucleotide sequences of the three genes we have chosen for regions of likely high susceptibility as targets to sdRNAs. The final selections are denoted in red and green segments (we used two colors only to be able to visualize easier, they are equally viable sequences) within the full sequences in Figures 1-3 below for the TAZ, MCT1 and IHH genes, respectively. All of these colored sequences for all three genes (A panels) were synthesized in the modified chemistries we are using for the sdRNAs—these extensive syntheses were all accomplished and we annealed the sdRNA sequences for the TAZ gene into viable double stranded sdRNA constructs. These were then used in the screens described below.

In the lower boxes (B panels) in these Figures 1-3 are the "target continuums" that have each of the target sequences with a given gene coupled in tandem to serve as a surrogate gene in the Dual Glo screens of the sdRNAs

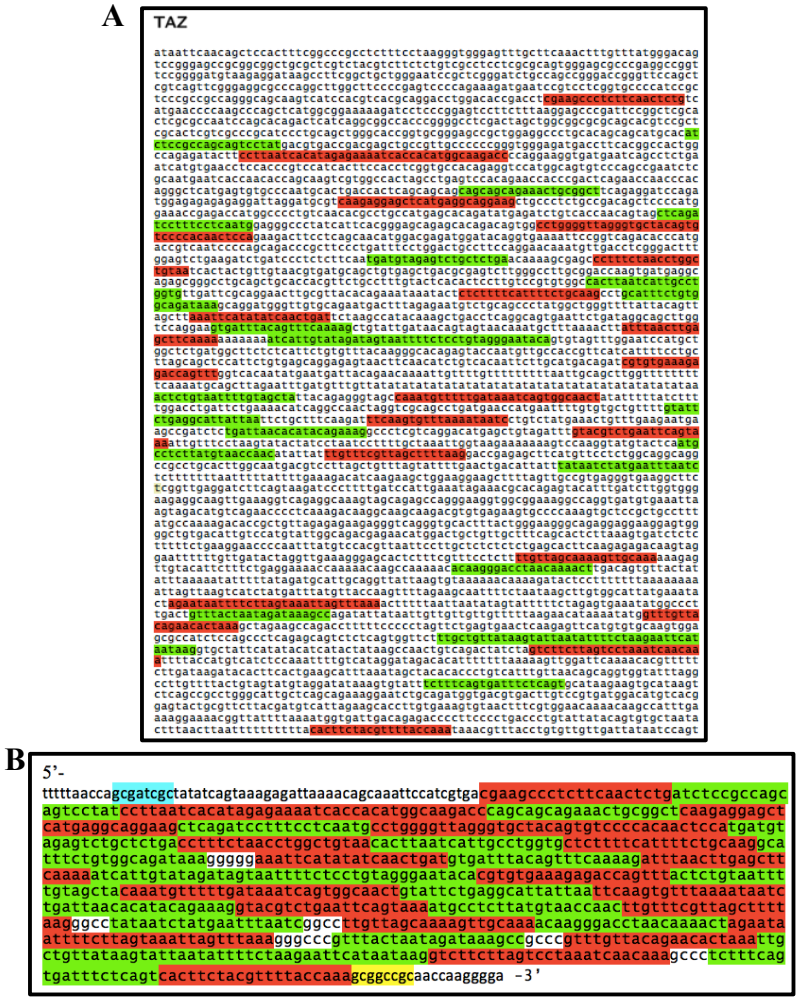


Figure 1: Target sites were identified and the "target continuum" insert for the Dual-Glo system was designed for TAZ.

A: Target sites (red and green colors) for sdRNA generation were identified according to parameters, such as Tm, GC content, hairpin formation possibility, non-specific binding, by using computational tools. B: The insert for the Dual-Glo system was designed by connecting the targeted regions back to back and by addition of restriction sites (SgfI for 5' and Not I for 3'). Parameters such as GC content and the size of the insert were optimized for efficient cloning. Designed insert was synthesized and used for screening.

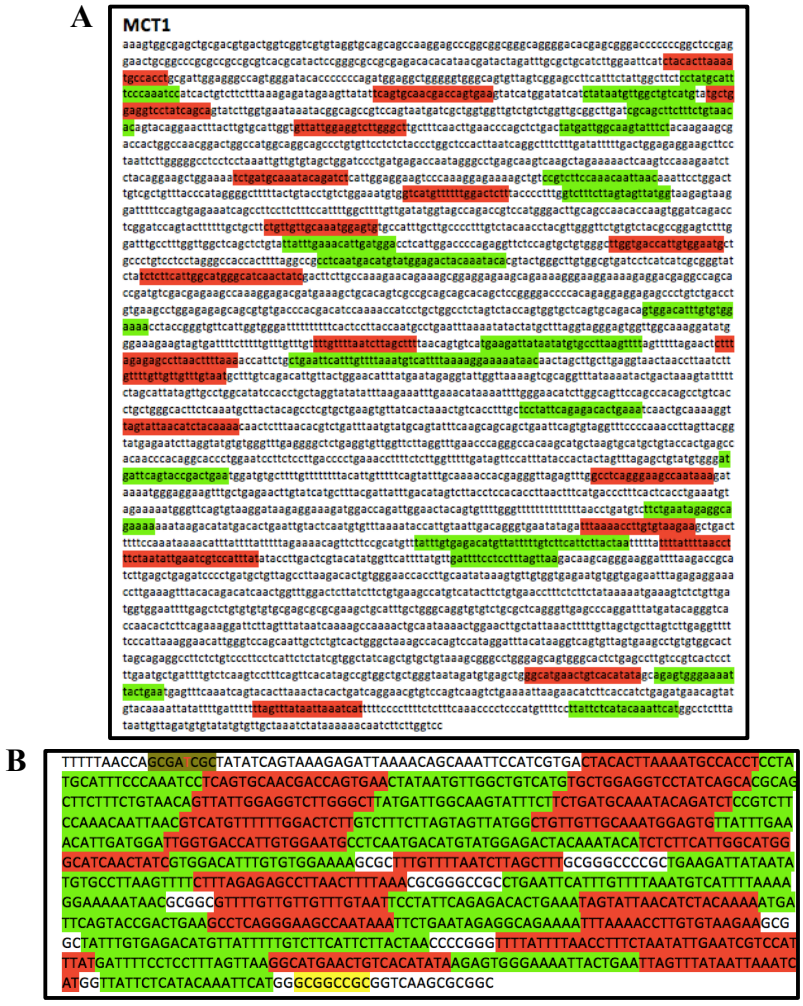


Figure 2: Target sites were identified and the "target continuum" insert for the Dual-Glo system was designed for MCT1.

A: Target sites (red and green colors) for sdRNA generation were identified according to parameters, such as Tm, GC content, hairpin formation possibility, non-specific binding, by using computational tools. B: The insert for the Dual-Glo system was designed by connecting the targeted regions back to back and by addition of restriction sites (SgfI for 5' and Not I for 3'). Parameters such as GC content and the size of the insert were optimized for an efficient cloning. Designed insert was synthesized and used for screening.

B

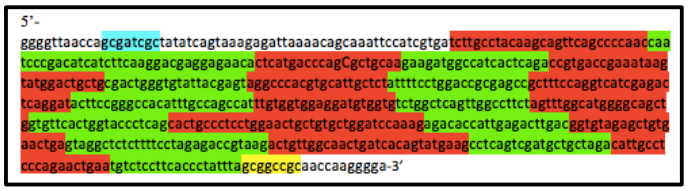


Figure 3: Target sites were identified and the "target continuum" insert for the Dual-Glo system was designed for IHH.

A: Target sites (red and green colors) for sdRNA generation were identified according to parameters, such as Tm, GC content, hairpin formation possibility, non-specific binding, by using computational tools. B: The insert for the Dual-Glo system was designed by connecting the targeted regions back to back and by addition of restriction sites (SgfI for 5' and Not I for 3'). Parameters such as GC content and the size of the insert were optimized for an efficient cloning. Designed insert was synthesized and used for screening.

To complete step 3 it is necessary to ligate the "target continuum" into an expression vector, denoted as psiCHECK-2 vector. Figure 4 shows the vector construct we have used to do this:
FIGURE 4:

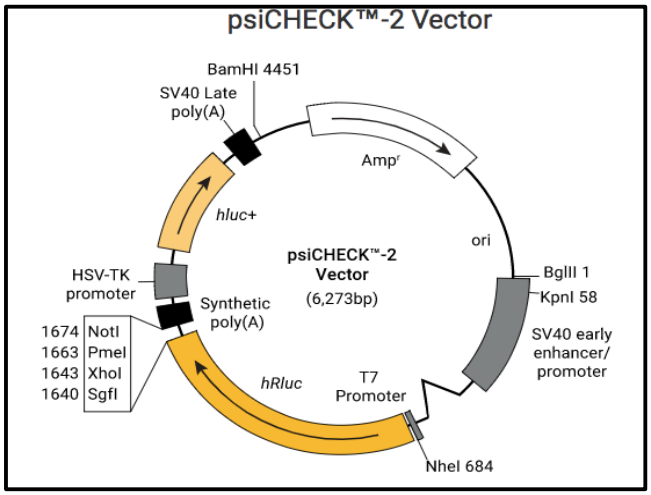


Figure 4*:* Overview of psiCHECK-2 vector for the screening of the sdRNAs. *psiCHECK-2 vectors are developed to enable faster and a quantitative way to screen RNA interference efficiency. It contains Renilla luciferase (hRluc) as the primary reporter gene in which decreased activity would be the indicator of the RNAi effect. Firefly luciferase(hluc+), on the other hand, acts as a normalization factor to increase the accuracy of the quantification. The multiple cloning site, which the designed insert would be cloned to, in the psiCHECK-2 Vector contains SgfI, XhoI, PmeI and NotI restriction sites.*

The mechanism of action of the psiCHECK-2 vector is schematized in Figure 5. The actual assay that we are currently using for our screens is:

- Culture mammalian cells in 10 cm dish in normal growth media.
- Transfect the cells with psi-CHECK vector with your designed insert.
- Administer sdRNA after 24hrs of transfection and seed the cells in 96-well plate.
- After 48-72hrs apply Dual-Glo Luciferase Assay.

Then we apply the Dual Glo Assay Protocol:

- 75 ul of Dual-Glo reagent, which has the substrate for the firefly luciferase in it, is added onto the cells. After 10 minutes to 1 hour incubation at room temperature, firefly luciferase activity is measured at 560nm. Firefly luciferase activity is used as the internal control of this assay.
- 75ul of Dual-Glo Stop&Glo reagent, which has the substrate for the Renilla luciferase in it, is added into the wells. After 10 minutes to 1 hr incubation at room temperature, Renilla luciferase activity is measured at 480nm. Renilla luciferase activity is determined and the knockdown efficiency of the candidate sdRNAs.

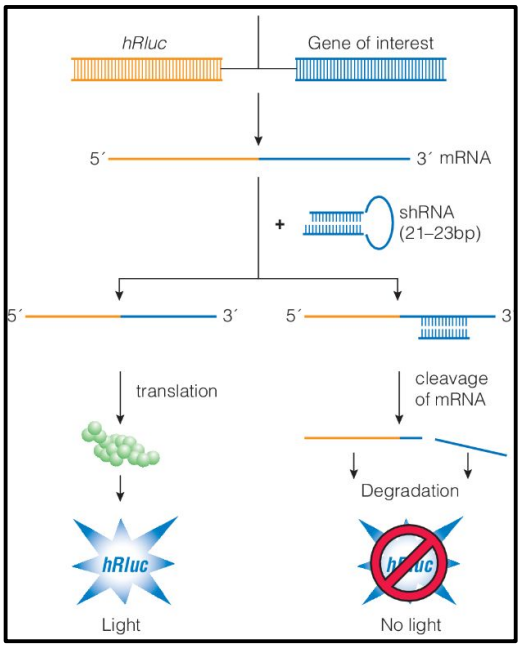


Figure 5: Mechanism of action of psi-CHECK vectors *Designed insert of gene of interest cloned at the 3' tail of the Renilla luciferase gene. Upon RNAi application, depending on how efficient RNAi silencing is, Renilla luciferase expression is either dampened (therefore the activity of it will be measured as decreased) or not changed (meaning RNAi failed to bind to the target sequence).*

It should be noted that we have also been successful with annealing the sdRNAs—Figure 6 provides a representative example of a set of these newly synthesized sdRNAs as double stranded constructs ready to be tested in the above assays.

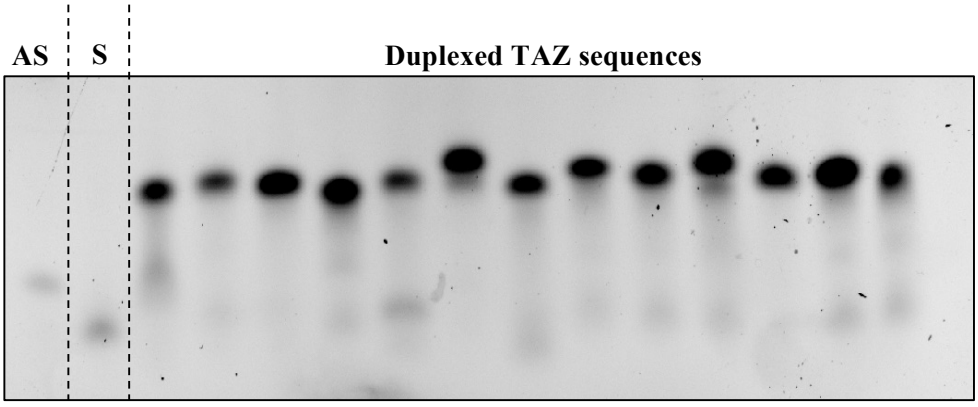


Figure 6 : Sense and anti-sense sequences duplexed successfully and confirmed by gel electrophoresis.

Equal moles of sense(S) and anti-sense (AS) strands are mixed and incubated at 95℃ 5 minutes. 25pmols of each reaction was run on a 20% TBE gel to confirm duplexing. Gel was stained with

By the end of the second quarter of project year 3, we had accomplished the first 4 steps of our strategy completely for both the TAZ and MCT1 target genes. **We then proceeded through the strategy:**

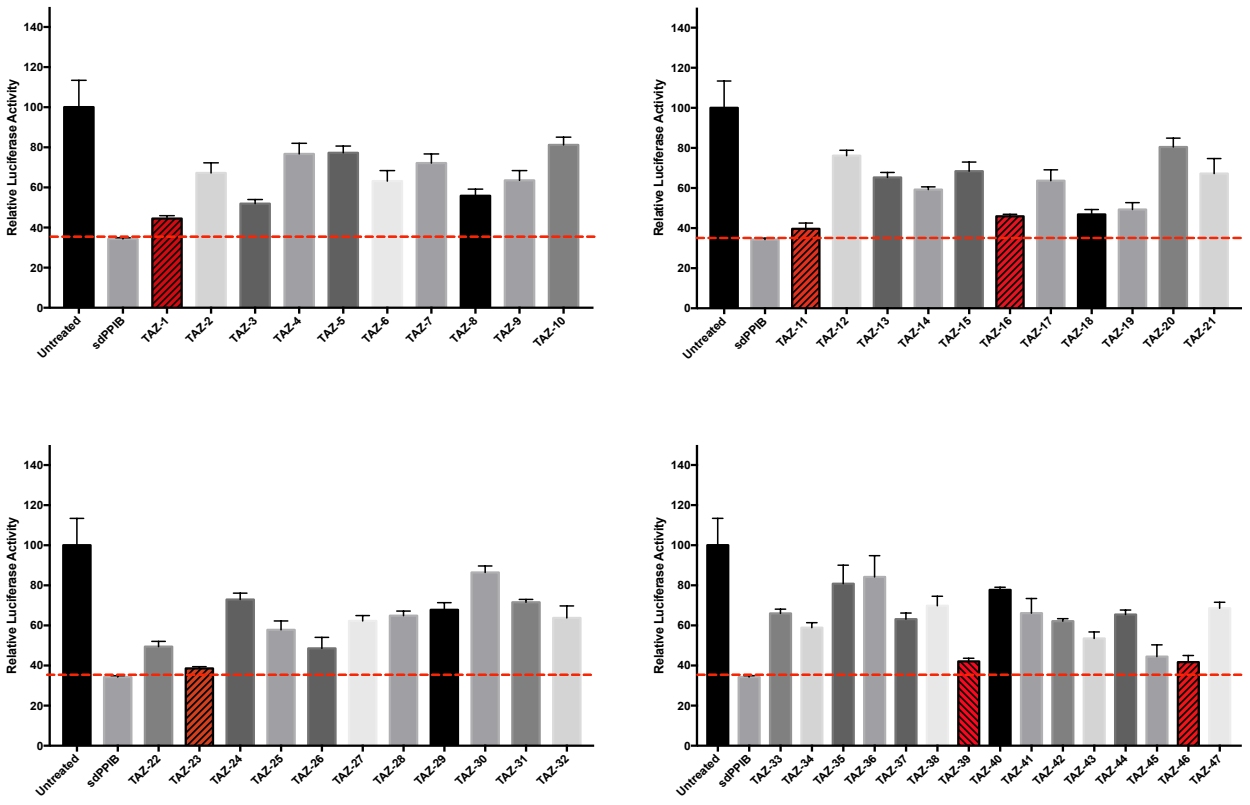
MCT1 sdRNAs all annealed: We had annealed the sdRNA sequences for the TAZ gene into viable double stranded sdRNA constructs, and we then completed manual annealing of all the 48 sdRNAs for the MCT1 gene.

TAZ and MCT1 sdRNAs all screened with the Dual Glo assay in intact 293T cells:

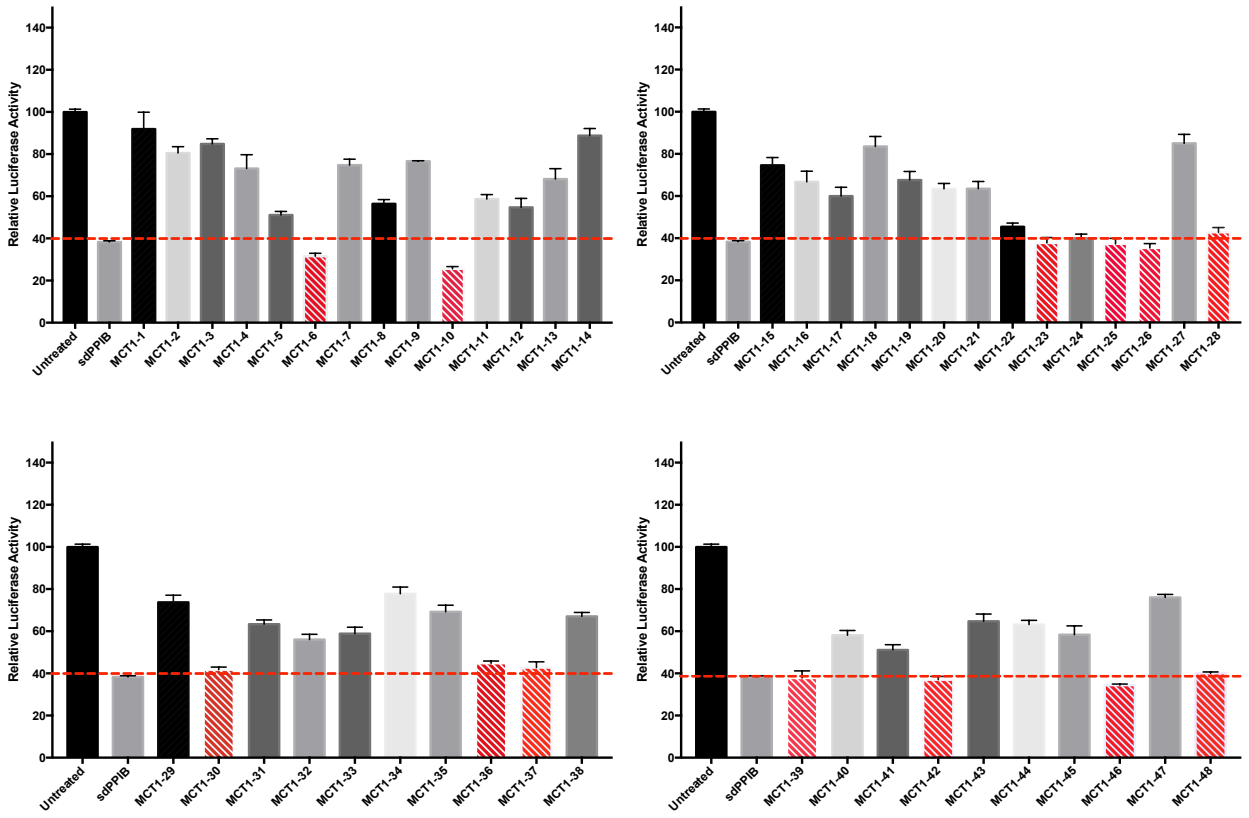
The cells were transfected with the psiCHECK vectors containing inserts for the target sequences for either the TAZ or MCT1 genes (plus the target sequence for the control PPIB gene), and were then used to screen with the 48 sdRNAs each against those genes. Several of the sdRNAs are directed to both mouse and human TAZ and MCT1 genes, as those sequence intervals are identical. Red bars in Figure 7 indicate very significant silencing that approaches or exceeds that for PPBI positive control.

Figure 7. *Screens for TAZ and MCT1 Chol-sdRNAs. For these experiments, 1.5uM sdRNA compound was used on 8k cells/well(transfected with related Dual Glo vector) in 96 well plates. 72 hrs later, the Dual-Glo assay was performed. The results indicate quantification of the Renilla luc./Firefly luc. Signal ratio.. (Firefly luciferase is acting as"housekeeping " since it is not effected by the silencing) and all data is normalized to the untreated group. PPIB sdRNA was used as a positive control, and consistently shows excellent silencing. A. TAZ screening. B, MCT1 screening. Experiments were run in quadruplicates. Red dashed line indicates the level of knockdown that has been obtained by using the positive control compound, sdPPIB.*

A



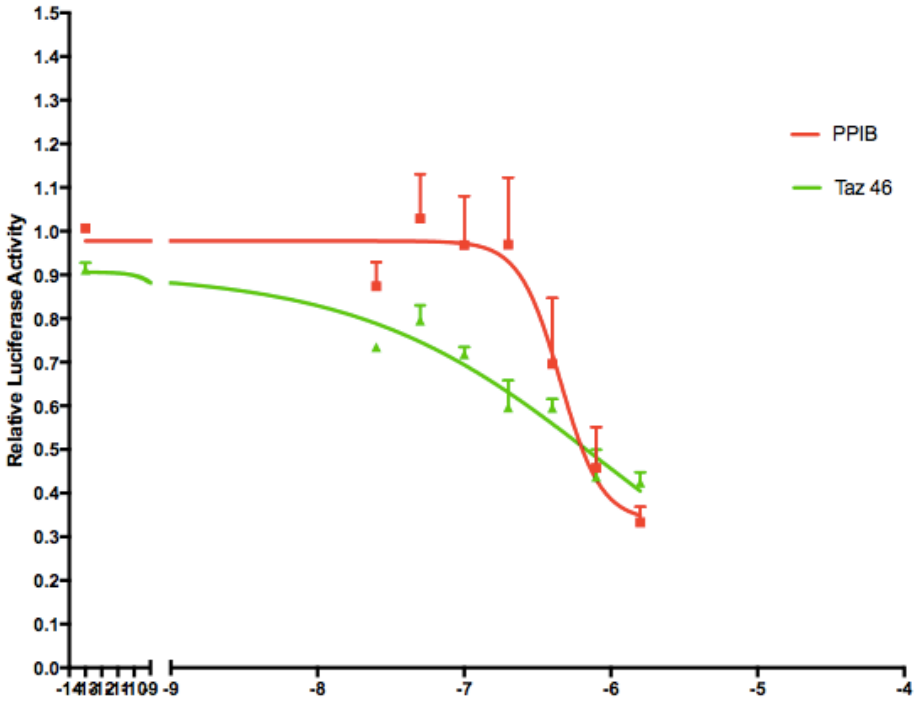
B



The assembled representative data in Figures 7A and 7B represents success in identifying a number of effective sdRNAs for MCT1 and TAZ in this screening format.

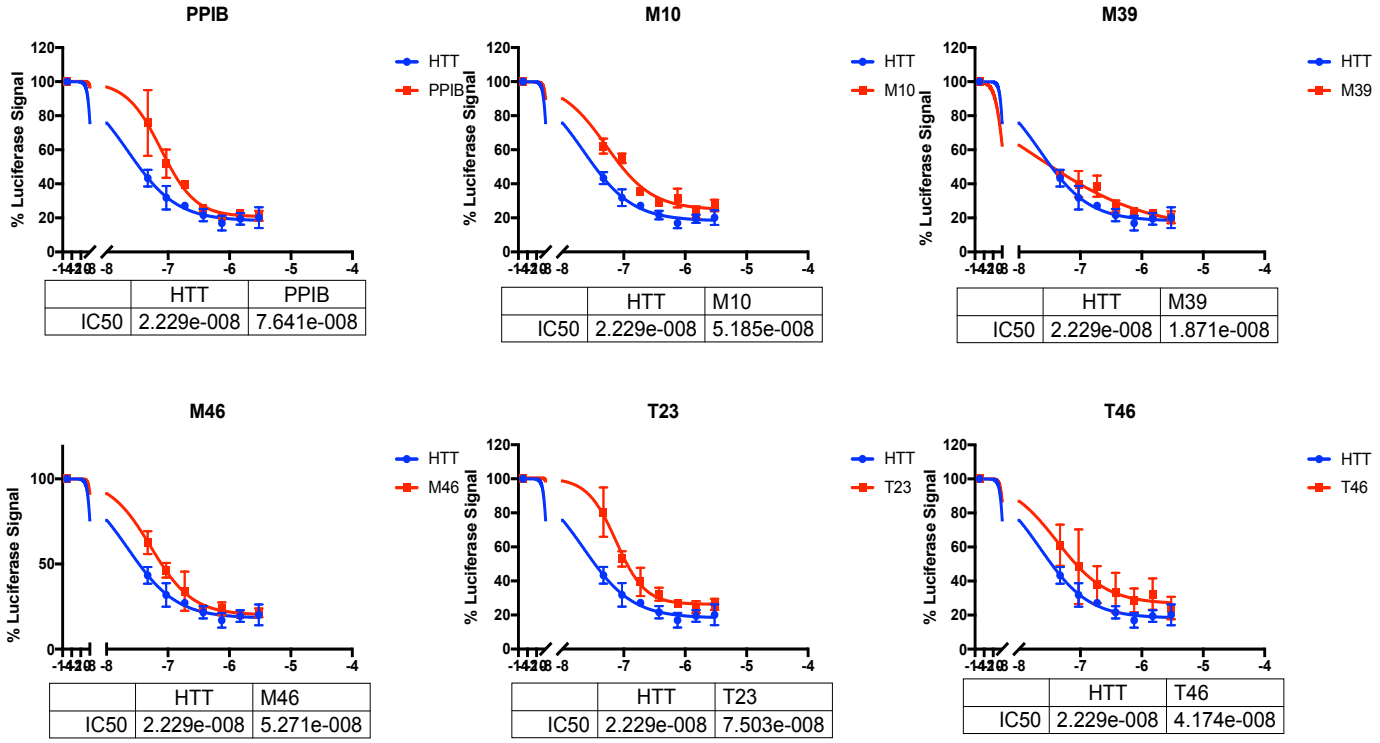
The next question on prioritizing these hits was to determine potency of the sdRNA hits. This was accomplished by using similar assay conditions, but with varying doses of the effective sdRNAs. The initial experiments were frustrating, as we found variable results as well as poor efficacy of the hits. An example of these initial data is shown below in Figure 8. We spent considerable effort in troubleshooting this problem, as all our previous results indicated that at least some of the sdRNAs should be high potency. After varying many parameters, we finally discovered that the high cell number may be a problem. This is due to some non-specific binding of the sdRNA to the cells, which skews the dose response relationship to higher values. **Once we discovered the solution to this problem, we obtained the data in Figure 9, which shows remarkably excellent dose response relationships for the most effective sdRNA hits against TAZ and MCT1.**

Figure 8(below): *Variable results for initial dose response experiments with high cell number. After transfection of 293T cells with the appropriate psiCHECK vector, various concentrations of the sdRNAs were added to 8000 cells per well.*



	PPIB	Taz 46
IC50	4.523e-007	8.427e-007

Figure 9(below): *Successful and reproducible dose response relationships for sdRNA hits against TAZ and MCT1 using lower cell numbers per well. Conditions same as in Figure 2, but with 4000 cells per well. HEK293 cells were transfected either with psiTAZ or psiMCT in 10cm-dishes. 24 hrs post-transfection cells were seeded into 96 well plates either with the sdTAZ compounds or sdMCT compounds at the final concentrations of 1500nM, 750nM, 375nM,187nM, 93nM, 46nM and 23nM. After 72 hrs incubation, firefly and renilla luciferase signals were measured via the Dual-Glo assay system. Experiments were performed in quadruplicates and sdPPIB was used as positive control. IC50 values were calculated via Non-linear regression analysis.*



	HTT	PPIB
IC50	2.229e-008	7.641e-008

	HTT	M10
IC50	2.229e-008	5.185e-008

	HTT	M39
IC50	2.229e-008	1.871e-008

	HTT	M46
IC50	2.229e-008	5.271e-008

	HTT	T23
IC50	2.229e-008	7.503e-008

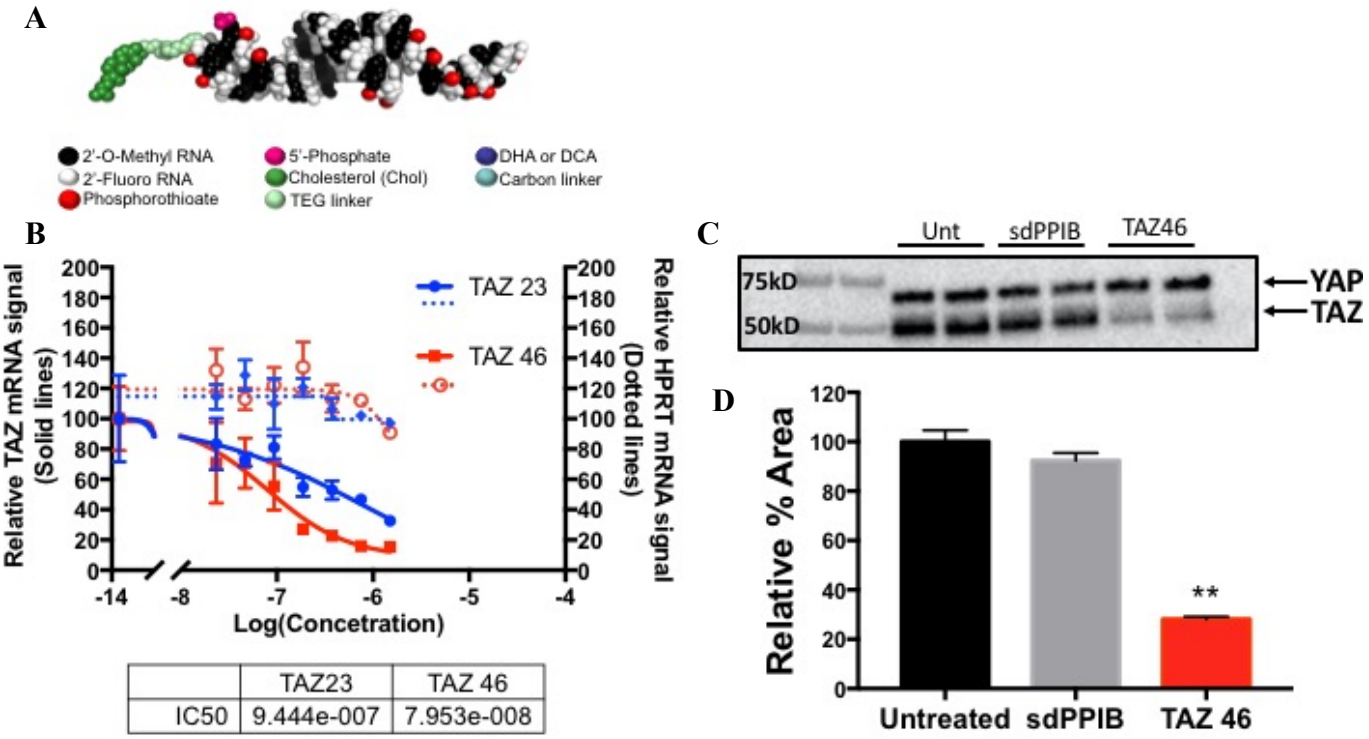
	HTT	T46
IC50	2.229e-008	4.174e-008

Based on the data of Figure 9 above, we were able to choose several sequences for sdRNAs against TAZ and MCT1 that we can now move forward to Steps 5 and 6 in our Strategy. These sequences are as follows:

Compund Name	Strand	Homology	Sequence
TAZ23	**AS**	Mouse/Human	**UGAUUUAUCAAACAUUUG**
TAZ23	**S**	Mouse/Human	**GUUUUUGAUAAAUCA**
TAZ46	**AS**	Mouse/Human	**UUUGGUAAAACGUAGAAGUG**
TAZ46	**S**	Mouse/Human	**CUUACGUUUUACCAAA**
MCT39	**AS**	Mouse/Human	**UUUAGUAAGAAUGAAGACAAA**
MCT39	**S**	Mouse/Human	**CUUCAUUCUUACUAA**
MCT46	**AS**	Mouse/Human	**UUCAGUAAUUUUCCCACUCU**
MCT46	**S**	Mouse/Human	**GGGAAAAUUACUGAA**

Experiments to demonstrate silencing of endogenous TAZ in cultured cells:

We initiated definitive experiments to show that the screens we have laboriously conducted actually yielded a highly active Chol-sdRNA that could silence the endogenous TAZ gene. This is shown in Figure 10 below:



	TAZ23	TAZ 46
IC50	9.444e-007	7.953e-008

Figure 10 : Potency screening of the selected compounds against the endogenous TAZ gene.
A: The structure of the Cholesterol conjugated and chemically modified siRNA. ***B:*** *8000 AML12 mouse hepatocytes per well plated in 96-well plate cell culture plate. The cells were treated with 7 different dilutions of 2 compounds targeting TAZ mRNA(TAZ23, TAZ46). After 72hrs of incubation with the compounds, mRNA levels of targeted gene, TAZ (straight line) and an internal control gene, HPRT (dotted line) were measured via Quantigene Singleplex gene expression assay. The screening revealed a highly potent compound that targets TAZ efficiently and specifically.* ***C,D:*** *To further confirm the efficient knockdown of TAZ we treated 3T3-L1 fibroblasts either with 1,5uM of sdPPIB or TAZ46. After 72hrs of incubation with the compounds, western blot analysis was carried out with 10ug protein from each sample. Western blot analysis showed a significant decrease in TAZ protein levels in TAZ46 treated group whereas no change in TAZ protein levels in sdPPIB treated group. YAP is the internal control for loading in the analysis.(**: p<0.005)*

Based on the data above, we concluded that TAZ46 was the best compound that could silence endogenous TAZ in cultured cells. The data for this conclusion is strong, as both mRNA (left panel) and protein blot (right panel) show significant silencing.

We therefore next sought to test whether the Chol-sdRNA TAZ46 compound could silence TAZ in liver in mice injected with this compound by subcutaneous route. The following protocol was used for this experiment, similar to what we established previously with test gene PPIB. For these experiments, we initiated large scale synthesis of the TAZ46 compound in the RNA Core Facility at UMASS Medical School.

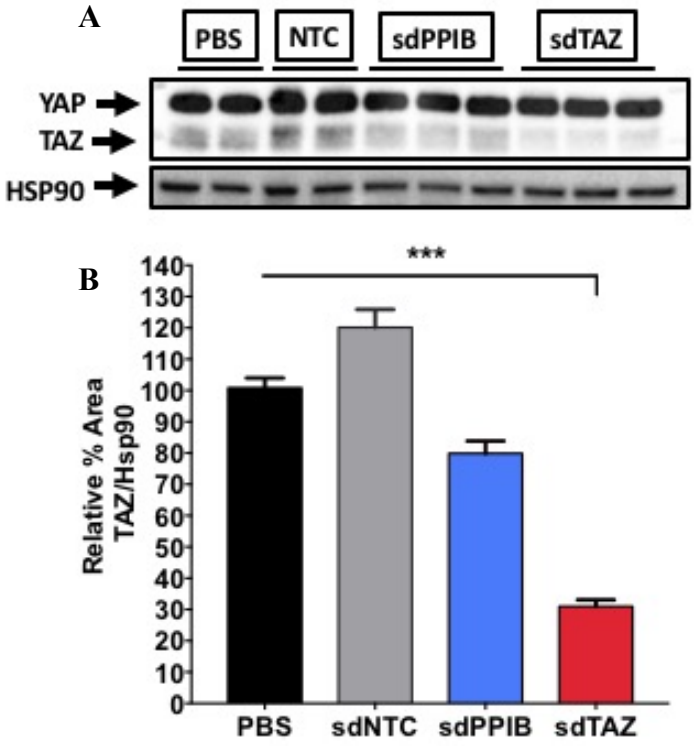


Figure 11: Silencing endogenous TAZ *in vivo following single subcutaneous injection of TAZ46.*
4 groups (n=6) of mice were injected with PBS, None-targeting control(NTC), sdPPIB, or sdTAZ (TAZ46) with the concentration of 10mg/kg via single subcutaneous injection.. Day-7 post injection mice were sacrificed and hearts, kidneys, spleens, subcutaneous adipose tissues and livers were harvested for further analysis. ***A, B:*** *To confirm the efficient knockdown in vivo, livers were processed and western blot analysis was carried out with 50ug whole liver protein from*

*each mouse. Western blot analysis showed a significant decrease in TAZ protein levels in sdTAZ injected group. HSP90 is the control for loading in the analysis. (***: p<0.0005)*

The above data in Figure 11 show a highly significant silencing of TAZ in the livers of mice injected with TAZ46 sdRNA. These data were quantified by careful assessment of the ratios of TAZ signal to HSP90 in each lane and then averaged and assessed for statistical significance. The small amount of TAZ silencing seen by PPIB knockdown is likely due to the slight anti-inflammatory effect of this test gene depletion.

Taken together, these data demonstrate we have reached the milestone of generating a Chol-sdRNA (TAZ46) that can silence a gene (TAZ) that has been shown to promote NASH in mouse models and likely in human disease. We are most encouraged to start using this compound, and others we can generate against TAZ and MCT1, in NASH mouse models to advance this technology to the clinic.

<u>Experiments demonstrating efficacy of Chol-sdRNA (TAZ) on a mouse NASH model:</u>

Based on the above success in silencing TAZ in liver with Chol-sdRNA, we sought to determine if this level of silencing was sufficient to provide a beneficial effect on NASH. We have started with a "prevention" protocol whereby treatment of mice with Chol-sdRNA (TAZ46) is begun along with the initiation of a NASH-causing diet (MCD diet in this experiment). This protocol is shown and described more fully in Figure 12, and the preliminary data we have obtained as of this date relates to the release of alanine transaminase activity (ALT) released from liver into the serum due to liver damage by NASH. Figure 13 shows the increase in ALT activity released into the serum upon treatment with MCD diet. Importantly, a dramatic effect of the Chol-sdRNA (TAZ) to reduce the serum ALT activity is observed at both 4 weeks and 7 weeks of diet compared to controls (PBS or non-targeting sdRNA). **These preliminary data demonstrate a strong beneficial effect of our tool compound Chol-sdRNA (TAZ46) on at least one parameter of NASH progression (ALT leakage from liver), a major milestone of this project.**

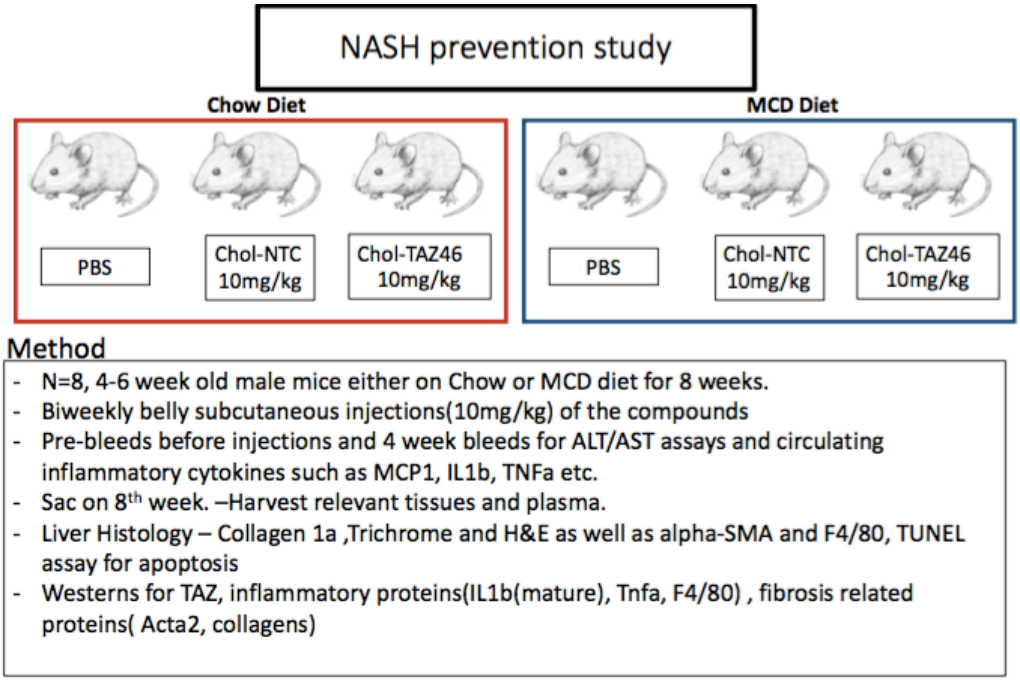


Figure 12: Experimental design and proposed methodology of NASH prevention study.
A: Experimental set up of the study. 6 groups (n=8) of mice were put on MCD diet and injected with PBS, Cholesterol conjugated None targeting control(NTC) , or Cholesterol conjugated sdTAZ with the concentration of 10mg/kg bi-weekly via subcutaneous injection from belly.

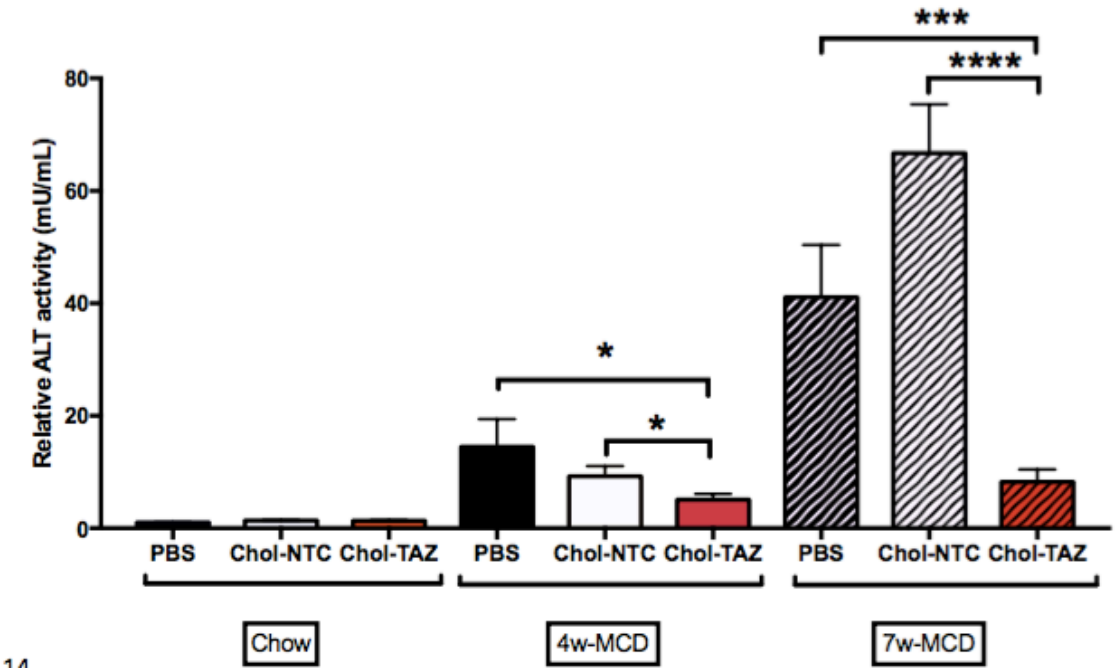


Figure 13: Liver damage caused by MCD diet was improved by subcutaneously injected Chol-TAZ Alanine Aminotransferase activity was measured at 4week and 7week time points. (* :$p<0.05$, ***: $p<0.0005$, ****: $p<0.00005$)

<u>Experiments demonstrating silencing of TAZ in hepatocytes by GalNAC-sdRNA:</u>

Since previous data has demonstrated that TAZ functions within hepatocytes (and not other cell types in the liver) to promote NASH, a more refined therapeutic strategy is to target only hepatocytes with GalNAC-sdRNA rather than all liver cells with Chol-sdRNA. We thus performed synthesis of the TAZ46 sequence in the GalNAC format to prepare GalNAC-sdRNA (TAZ). We then treated mice with either PBS, non-targeting GalNAC-sdRNA, or GalNAC-sdRNA (TAZ) with single subcutaneous injection (Figure 14). As shown in the Figure, we achieved highly significant silencing of TAZ in the livers of the latter group compared to the other control groups of mice. **These data demonstrate achievement of another major milestone in the project: identification of a tool GalNAC-sdRNA compound that can potently silence TAZ <u>selectively in hepatocytes</u> following subcutaneous injection in mice.**

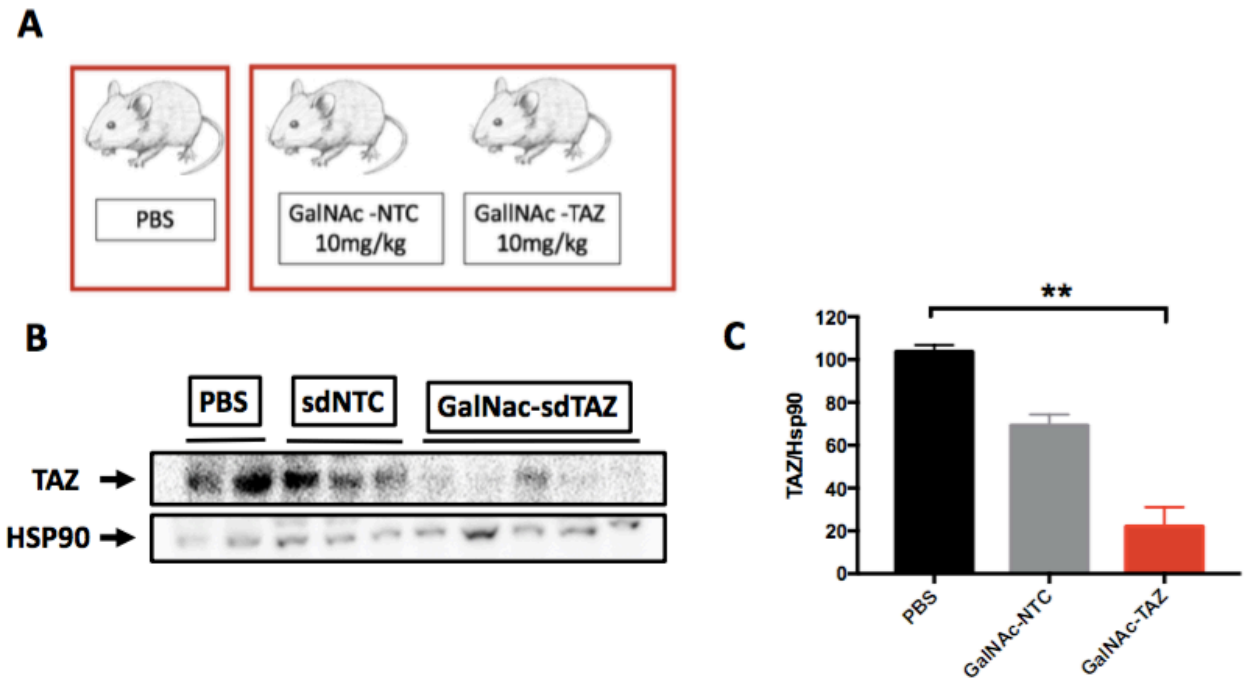


Figure 14: In vivo knockdown with GalNAc conjugated-TAZ46 compound
A: Experimental set up of the study. 3 groups (n=6) of mice were injected with PBS, GalNAc conjugated none targeting control(NTC) or GalNAc conjugated sdTAZ with the concentration of 10mg/kg via single subcutaneous injection from belly. 3 weeks post injection, mice were sacrificed and hearts, kidneys, spleens, subcutaneous adipose tissues and livers were harvested for further analysis. **B,C:** To confirm the efficient knockdown in vivo, livers were processed and western blot analysis was carried out with 50ug whole liver protein from each mouse. Western blot analysis showed a significant decrease in TAZ protein levels in sdTAZ injected group. HSP90 is the control for loading in the analysis. (**: $p<0.005$)

Opportunities for training and professional development: This project offers excellent training for the graduate student and postdoctoral fellows working on it.

Dissemination to communities of interest: Nothing to report

4. Impact:

Impact on principle discipline: Novel chemistries for delivery of siRNA established and publications on our work from this project will be forthcoming.

Impact on other disciplines: Nothing to report

Impact on technology transfer: Key Patent filings contemplated next year.

Impact on society: Progress towards development of a therapeutic on schedule.

5. CHANGES/PROBLEMS

This project continues to focus on the original goal of developing a tool compound(s) for chemically modified, self delivery siRNA of the Chol-sdRNA or GalNAc-sdRNA type (or both) that will silence the target genes TAZ, MCT1 or IHH and alleviate NASH in mouse models of this disease. **We are poised to accomplish this final goal, with the exciting data we have produced, and are hopeful we can obtain funding through the DOD EXPANSION program to bring these studies forward to an IND and clinical trials.**

6. PRODUCTS: Nothing to report

7. PARTICIPANTS and OTHER COLLABORATING ORGANIZATIONS

Individuals who worked on the project:

Name: Michael P. Czech
Project Role: PI
Researcher Identifier (e.g. ORCID ID): NIH/eBRAP ID: MPCZECH
Nearest person month worked: 2.4

Contribution to Project: As PI, Dr. Czech directed, designed and analyzed research.

Name: Batuhan Yenilmez
Project Role: UMass Graduate Student
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 12
Contribution to Project: Batuhan performed in vitro and in vivo experiments with sdRNA against the test gene PPIB and RIP140.

Name: Yuefei Shen
Project Role: Post Doc
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 7
Contribution to Project: Dr. Shen designed and synthesized GeRP formulations and carried out in vitro assessments.

Name: Sarah Nicoloro
Project Role: Res. Associate
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 6
Contribution to Project: Ms. Nicoloro provided technical assistance on all aspects of this project.

Name: Mark Kelly
Project Role: Research Associate I
Researcher Identifier (e.g. ORCID ID) n/a
Nearest person month worked: 5
Contribution to Project:

Changes in PI current support: Nothing to report

Other Organizations: Nothing to report

8. SPECIAL REPORTING REQUIREMENTS: N/A

9. APPENDICES: N/A