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14. ABSTRACT

This project seeks to use CRISPR-based deletion of specific target genes such as RIP140 in mouse and human adipocytes as a means to enhance "browning" and energy expenditure in these cells. Once this is achieved, the goal is to transplant such genetically modified, metabolically activated mouse or human adipocytes into normal or "humanized" mice, respectively, to enhance systemic glucose tolerance in diabetic animals. During this first year an exciting project achievement has been to develop and advance methods to optimize the efficiency of RIP140 gene deletion from about 50% (which was the status at time of proposal) to nearly 100%. This remarkably opportune advance positions us to progress toward our goals of alleviating diabetes in mice more rapidly than anticipated, and to quickly test the effects of deletions of other target genes that may also provide increased metabolism prior to transplant. We have thus exceeded our SOW goals for this first year by identifying RIP140 sgRNA that provides nearly 100% gene deletion in mouse adipocytes by CRISPR without the need for subcloning cells, and currently applying this advance to human adipocytes as proposed.

15. SUBJECT TERMS

Adipocytes, metabolism, uncoupling protein, brown adipose tissue, CRISPR, RIP140, energy expenditure, type 2 diabetes, obesity, glucose tolerance

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1. INTRODUCTION:

Type 2 diabetes is a debilitating disease in millions of patients including US veterans. While obesity is the major driver of type 2 diabetes and is extremely difficult to effectively treat, a significant number of obese individuals are metabolically healthy and remain diabetes-free, showing that obesity without diabetes is physiologically possible. It is not clear how this occurs, but it does correlate with a higher distribution of subcutaneous white adipose tissue (sWAT), and with higher levels of brown adipose tissue (BAT). BAT contains brown adipocytes loaded with mitochondria that can be uncoupled by high expression of the uncoupling protein UCP-1 to produce heat and expend energy plus secrete beneficial factors. Brown-like or "Beige" adipocytes also occur in sWAT when humans or mice are cold exposed. Our approach is based on the goal of enhancing adipose tissue energy expenditure and secretion of beneficial factors through an adipocyte "browning" process whereby UCP-1, mitochondrial fatty acid oxidizing enzymes and secreted factors that enhance glucose tolerance are upregulated by CRISPR-based target gene deletion. Thus, converting fat-storing sWAT to fat-oxidizing BAT which secretes beneficial "BATokines" by deleting genes that suppress energy metabolism is our goal.

To attack this problem, we proposed to further develop CRISPR delivery particles that can be administered *in vitro* (i.e., *ex vivo*, prior to adipose re-implantation) or *in vivo* to delete a gene(s) that controls "browning" of WAT. We target the Nrip1 gene (also denoted RIP140) with CRISPR-based deletion in mouse and human adipocytes, which from our previous work causes dramatically enhanced fat oxidation, energy expenditure and beneficial "adipokine" secretion. Importantly, knockout of RIP140 alleviates type 2 diabetes in mice. Thus, the ultimate goal in this collaboration between the Michael Czech and Silvia Corvera laboratories at UMASS Medical School is to enhance human WAT "browning" by CRISPR-based targeting of RIP140 and testing the "Beige" adipocytes on systemic glucose tolerance when implanted into "humanized" mice.

2. KEYWORDS:

Adipocytes, metabolism, uncoupling protein, brown adipose tissue, CRISPR, RIP140, energy expenditure, type 2 diabetes, obesity, glucose tolerance

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

There has been one key overall goal for project months 1-12 as defined in Specific Aim 1 and in the original SOW: To identify sgRNAs that drive maximal CRISPR-mediated deletion of the metabolism-suppressing gene Nrip1 (RIP140) in primary mouse adipocytes from sWAT (Czech lab) and in human beige adipocytes (Corvera lab).

This overriding goal was subdivided into 6 subtasks in our SOW:

1. Prepare Cas9 protein and endoporter protein. 1-3 months-**ACCOMPLISHED Site 1**
2. Generate RIP140 sgRNA sequences for the mouse and human genes. 3-6 months-**ACCOMPLISHED Sites 1 and 2**
3. Obtain HRPO/ACURO approvals. 1-3 months—**ACCOMPLISHED Sites 1 and 2**
4. Obtain local IRB/IACUC approvals. 1-3 months—**ACCOMPLISHED Sites 1 and 2**
5. Testing RIP140 sgRNAs in mouse adipocytes in vitro. 3-12 months—**ACCOMPLISHED Sites 1 and 2**
6. Testing RIP140 sgRNAs in human adipocytes in vitro. 3-12 months-**ACCOMPLISHED and in progress for optimization. Sites 1 and 2**

As described in detail below in the Accomplishments Section, these objectives have been superseded by our unexpected discovery that application of electroporation to preadipocytes mixed with CRISPR/sgRNA complexes raises efficiencies of RIP140 gene deletion to nearly 100%. This has allowed us to rapidly test effects of deletion of other genes (e.g., TGFbR1 and NCOR1) on the adipocyte "Beiging" process, and most importantly enables the potential to perform double or triple gene deletions for super activation of metabolism and beneficial adipokine secretion.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Accomplishments for each of the subtasks in 1-12 months SOW are itemized below.

Subtask 1. Prepare Cas9 protein and endoporter protein.

Comparison between commercially available versus in house prepared/purified Cas9 protein. A key element and advantage of our approach is the avoidance of vectors and vector-based expression of Cas9 and sgRNA for mediating CRISPR-induced gene deletion in preadipocytes. The reason is the potential toxicities of using vectors, which have the potential to cause immune reactions and eliminating the gene therapy that is applied. Therefore, our approach uses the Cas9 protein itself and synthesized sgRNA as the CRISPR-mediating reagents, and these are degraded within cells prior to re-implantation of the adipocytes back into mice or humanized mice. We use our method to form complexes of Cas9 protein, sgRNA and Endoporter peptide (denoted CriPs) to directly incubate with preadipocytes *ex-vivo.* **This requires us to prepare or obtain Cas9 protein, sgRNA and Endoporter in high quantities for reasonable cost, and we have investigated 2 ways to do this:**

A. Commercial availability. Cas9 Protein

Cas9 protein is a Type II effector protein from *Streptococcus pyogenes* (SpCas9). Our lab has developed a transfection complex of ribonucleoproteins (RNPs) using SpCas9 purchased from

PNA Bio with NLS (nuclear localization signal) conjugated to the protein. It is a stable protein that has a half-life in cells of approx. 24hr. PNA Bio was one of the first companies to prepare purified Cas9-NLS protein for purchase. By purchasing purified Cas9 protein to make RNP complexes we were able to prepare RNP complexes early in the field of CRISPR knockdown. We have achieved excellent results with PNA Bio Cas9 protein that comes in a form that has a high purity and is devoid of endotoxin. We purchase the high concentration, lyophilized form that is available at a concentration of 250ug/tube. We have a price quote from PNA Bio that gives us a discount when purchasing larger amounts of the nuclease. The cost we pay per 250ug tube is $262.50 which is approx. 30% discounted.
https://www.pnabio.com/products/CRISPR_Cas9.htm

B. Commercial availability, Endoporter peptide

We purchase Endoporter from Gene Tools Inc. We have been preparing our CRiPs with Endo-Porter Aqueous that is at 1mM in a solution of mannitol. Endoporter is a novel peptide that allows for delivery of substances into the cytosol of cells by an endocytosis-mediated method. Once endocytosed, the Endoporter and cargo exit the endosome into the cytosol. For our studies we have utilized Endoporter to complex with sgRNA and Cas9 protein to make RNP complexes. Endoporter has also been shown to be effective at transfections with up to 10% serum that is a unique feature of this transfection reagent. Endo-Porter is an amphipathic α-helical peptide with one face composed predominantly of aliphatic lipophilic amino acids, and the other face composed of basic amino acids. (Summerton J. E. 2005, pg. 18 of patent 587287-UM9-216-1). It is important to note that our new electroporation method (as of 05-01-2019) for delivery of Cas9 protein and sgRNA into preadipocytes DOES NOT require Endoporter protein, as our original method did. Therefore, the experiments reported in sections 5 and 6 of our Accomplishments below do not use Endoporter protein. However, we may use Endoporter in the future if we find it beneficial to combine the electroporation method of delivery with Endoporter.

C. In house-produced Cas9 protein.

Since the first discovery of CRISPR technology and gene editing, *Streptococcus pyogenes* (Sp)-Cas9 has been the primary product that has been investigated to determine the accuracy, efficiency and safety of its' enzyme based gene editing. Through this work have come alternate enzymes that work in a similar fashion to SpCas9. Our Medical School has been at the forefront of this work with several labs pursuing this technology and alternate enzymes to SpCas9 for use in CRISPR technology. Additional species that produce effective Cas9 enzymes such as *Streptococcus pyogenes* (Sp), and *Neisseria* meningitides (Nm) have been discovered, and there are newly discovered forms of CRISPR enzymes such as Cpf1 and Cas12a that are promising in terms of specificity, efficacy and safety. While spCas9 nuclease is still the most widely used, the accuracy of wild type spCas9 is limited. The need for improved spCas9 proteins has driven the work described in a recent publication by Scot Wolfe, a professor in our Department of Molecular Cell and Cancer Biology. The Wolfe lab has developed chimera Cas9 proteins that have been shown to be highly specific to the target gene and produce accurate cutting with high efficiency (Bolukbasi MF et al Nat Commun. 2018 Nov 19;9(1):4856. PMID: 0451839). The chimera Cas9 proteins consist of two Cas9 proteins from the same species $SpCas9^{WT}$-$SpCas9^{WT}$ and dual nuclease formats with $SpCas9^{WT}$-Sa/$NmCas9^{WT}$. These fusion proteins have been shown to have a higher specificity to the target DNA sequence, in addition to having a higher frequency of cutting. We plan to take advantage of this emerging technology and to test these modified Cas9 nucleases to further our efficacy and specificity in future experiments.

We have investigated spCas9 generated by Scot Wolfe's lab in experiments side-by-side with the commercially available PNA Bio Cas9 to determine relative efficacy and compare cost. **The objective of these experiments was to determine whether in-house prepared Cas9 protein could be as efficient and as devoid of endotoxin as commercially prepared Cas9 as a means of future cost savings in this project.** The data presented below in Figure 1 show results of a representative experiment showing the efficacy of CriPs prepared with either the commercially available Cas9 versus the Wolfe lab produced Cas9. Primary preadipocytes obtained from GFP expressing mice were used in these experiments and CriPs were prepared with sgRNA directed against the GFP gene, as a test gene.

The experiment shown in Figure 1 shows the best efficiency of GFP gene deletion obtained with commercial Cas9 was 60% (at the 25uM Endoporter, EP, concentration), while the in-house Cas9 preparation only gave 31% efficiency of GFP gene deletion. A second experiment shown in our semi-annual technical report (not shown here) with a more recent, more purified preparation, of Cas9 prepared in-house on the other hand shows a better efficiency of GFP gene deletion as does the commercial preparation. This was achieved at 20uM Endoporter concentration and using PBS rather than Buffer 3 under the conditions of this experiment. **Taken together, these experiments have shown us that we can prepare in an academic lab setting Cas9 that is highly effective in mediating gene deletion when packaged in CriPs using our standard technology. This is an important advance, and we now conduct our experiments mostly using Cas9 prepared by the methods of the Wolfe lab. This will save substantial costs for obtaining Cas9.**

FIGURE 1

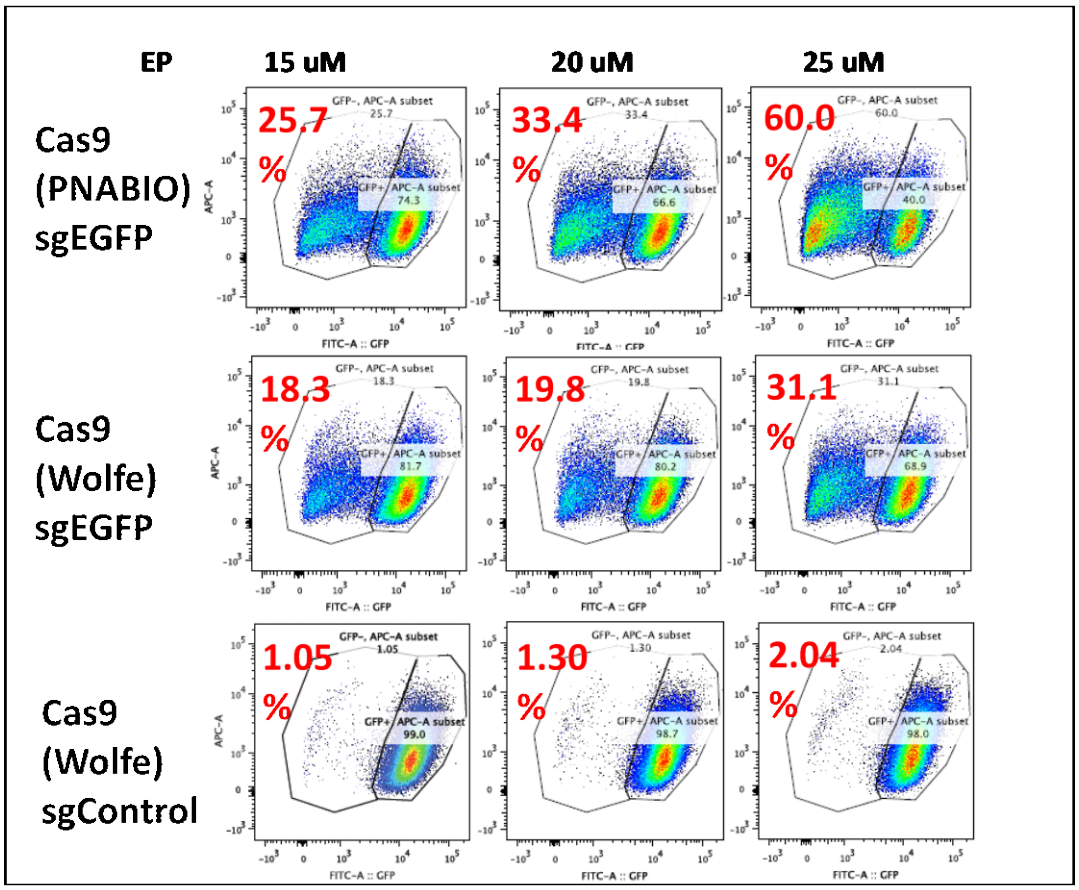

Figure 1. Primary pre-adipocytes harvested from GFP-heterozygous mice, treated in vitro with CRiPs prepared with PNA Bio Cas9 **versus** Wolfe lab-prepared Cas9 {Cas9/sgRNA (100nM, 1:1)}, using our published **Buffer 3** conditions and Endoporter peptide(EP). EP concentrations are 15, 20 or 25uM. Cells were FACS sorted 5 days post transfection to visualize the extent of loss of GFP expression (movement of fluorescent signal to the left), which signifies deletion of the GFP gene. Percentages in Red Font indicate the percent loss of GFP signal.

Subtask 2. Generate RIP140 sgRNA sequences for the mouse and human genes.

Prior to analyzing DNA sequences for optimum sgRNA target sequences we performed a literature search on the most up to date, effective methods at designing guide sgRNA's for CRISPR. There are several recommended sgRNA tool design websites available such as Broad GPP, Deskgen, Benchling.com, CasOFFinder, CHOPCHOP. The current web based sgRNA guide selection tool that we have utilized is the free platform developed by the Broad Institute called the GPP (Genomic Perturbation Platform). This design tool has incorporated the Rule Set 2 method from Doench, Fusi et. al., Nature Biotechnology 2016, that ranks and chooses candidate sgRNA sequences for the targets by priortizing the highest on-target activity and the lowest off-target activity. In addition, other considerations we employ are to avoid choosing sgRNA sequences that code for amino acids near the N' terminus or C' terminus. In general, we also follow the suggested rule that we choose a guide RNA that falls in the 5-65% of the protein coding region.

In addition, we have also used the commercial tool from Synthego. Details from their site are: The Synthego CRISPR Design Tool sorts the guides by using several heuristics, such as cut site on the gene, common exon, and high activity. The CRISPR Design Tool preferentially chooses sequences that occur in earlier exons as an indel in these regions will improve the chances of knocking out the gene's function. It then preferentially selects guide sequences in exons that are common to various splice variants. Finally, the CRISPR Design Tool uses on-target score (Doench et. al, 2016) and off-targets as a pass/fail criteria to select guide RNA sequences that we recommend for use to knockout a gene.

The IDT sgRNA design tool uses a proprietary logarithm to pick the best guide sequences. Often they are in the same exon that Synthego CRISPR Design Tool chooses. Thus far, they have proven to be as efficient for knockdown percentage as the Synthego designs. We show below in Table 1 a number of RIP140 sgRNAs (previously presented in our semi-annual report) and, in Table 2, 12 human-based sgRNA's designed for RIP140 that we are beginning to test in human preadipocytes and adipocytes. Some of the methods used to choose effective guide RNA's are focused on designing the guide in the most common exon in all variants, and choosing a guide that is in an earlier exon.

Table 1. Single guide RNA (sgRNA) for Mouse Nrip1

Mouse Target Gene Symbol	sgRNA sequence	PAM sequence	Early Exon	Common Exon	On target score	Off targets/ Off target score	Rank	Algorithm used
Nrip1	CTTGTATTGAACATGACTCA	TGG	No	Yes	0.424	0,0,0,5,84	1	Synthego
Nrip1	ATGACTCATGGAGAAGAGCT	TGG	No	Yes	0.495	0,0,0,3,55	2	Synthego
Nrip1	GCTTGGCTCTGATGTGCATC	AGG	No	Yes	0.510	0,0,0,0,73	3	Synthego
Nrip1	ATTGTCTTAACTTACCTCGA	AGG	No	Yes	0.453	0,0,0,11, 136	4	Synthego
Nrip1	TTGTCTTAACTTACCTCGAA	GGG	No	Yes	0.560	0,0,0,18, 198	5	Synthego
Nrip1	TTACTAATGCATCAGGCAGC	AGG	No	Yes	0.585	0,0,0,10, 119	6	Synthego
Nrip1	GATGCATTAGTAACCCTTCG	AGG	No	Yes	53	87	1	IDT
Nrip1	GTCAGTACCCAGACGTACCA	GGG	No	Yes	56	85	2	IDT
Nrip1	ATAAGGTTTGGAGTCACGTC	AGG	No	Yes	78	84	3	IDT
Nrip1	GGATTTAAGGTGCTATGGCG	TGG	No	Yes	53	81	4	IDT

Nrip1	CGTAAAGAAGGAAGCGTTGC	TGG	No	Yes	51	81	5	IDT
Nrip1	AGGACTGGAACGCGGCAAAG	CGG	No	Yes	55	66	6	IDT

Table 1. The above sgRNA sequences are new sequences designed by two different algorithms for chosing sgRNA; Synthego CRISPR Design Tool and IDT sgRNA design tool. Both algorithms have proven to be very effective at knocking down genes.

Table 2. Single guide RNA (sgRNA) for Human Nrip1

Human Target Gene Symbol	sgRNA sequence	PAM sequence	Early Exon	Common Exon	On target score	Off targets/ Off target score	Rank	Algorithm used
Nrip1	CTTCTATTGAACATGACTCA	TGG	yes	yes	0.576	0,0,0,10,107	1	Synthego
Nrip1	ATTGTTTTAACTTACCTAGA	TGG	yes	yes	0.560	0,0,1,19,206	2	Synthego
Nrip1	GCTTGGCTCTGATGTGCACC	AGG	yes	yes	0.511	0,0,1,7,171	3	Synthego
Nrip1	AAGTTAAAACAATAGAATCC	TGG	yes	yes	0.526	0,0,0,26,416	4	Synthego
Nrip1	ATTGTTTTAACTTACCTAGA	AGG	yes	yes	0.408	0,0,0,11,199	5	Synthego
Nrip1	TTACTAATGCATCAGGCAGC	AGG	yes	yes	0.454	0,0,0,8,91	6	Synthego
Nrip1	ACATCAGGAAGATTCGTATC	AGG	no	yes	61	78	1	IDT
Nrip1	ATGGTTGACAGTGTGCCTAA	AGG	no	yes	64	70	2	IDT
Nrip1	AACTGGACCATTACTTTGAC	AGG	no	yes	74	68	3	IDT
Nrip1	GTCATGTGCTGCAAGATTAC	AGG	no	yes	65	67	4	IDT
Nrip1	CTCGAGAATACTGCTGCAAA	TGG	no	yes	53	65	5	IDT
Nrip1	AGAAGGATTACTAATGCATC	AGG	no	yes	76	52	6	IDT

Table 2. The above sgRNA sequences are new sequences designed by two different algorithms for choosing sgRNA; Synthego CRISPR Design Tool and IDT sgRNA design tool. Both algorithms have proven to be very effective at knocking down genes.

Subtask 3. Obtain HRPO/ACURO approvals.

These approvals were obtained and confirmed by emails.

Subtask 4. Obtain local IRB/IACUC approvals.

These approvals were obtained and confirmed by emails, and the 3 year UMASS Medical School IACUC renewal for Czech laboratory site 1 was approved on May 22, 2019 and forwarded to DOD officers on May 22, 2019. IACUC approval for site 2 continues for this period as well.

Subtasks 5 and 6. Testing RIP140 sgRNAs in mouse and human adipocytes in vitro.

The major stumbling block in our project has been the level of efficiency of KO (about 50%) that we were able to obtain with CRISPR-based Crips when we initially submitted our proposal. We

had indicated that one way around this technical obstacle was to perform single cell cloning, starting with single cells that had been transfected with Crips. This is a tedious process that may not work, as preadipocytes that are passaged or that undergo many rounds of division usually lose their ability to differentiate to adipocytes with high efficiency. This clearly brought a higher level of risk to our project than is desirable, and we have worked hard to eliminate this risk.

We are now delighted to report a major breakthrough in our project, which allows us to obtain near 100% efficiency of Cas9-mediated disruption of genomic DNA in preadipocytes. Thus, for our *ex vivo* strategy in this project over the next 2 years, we will be able to ultimately implant into mice adipocytes that are nearly all harboring disrupted expression of one or more target genes. Our lab has been fortunate to work in close proximity to several leaders in the field of CRISPR/Cas9, including Professors Scot Wolfe and Eric Sontheimer. We recently adapted techniques of transfection using methods of electroporation the Sontheimer Lab has employed involving the **Neon System** manufactured by Thermofisher Science. Similar to our Crip nanoparticles, this method can be used for the purpose of transfecting Cas9 protein and sgRNA to deliver the RNP (ribonucleoprotein) complexes directly to the cells without the need for Endoporter.

By refining such electroporation methods to suit our cell type (preadipocytes), we have succeeded in obtaining very high efficiencies of gene disruption (85-98% range). Briefly, we prepare the RNP complexes first by combining the manufacture's buffer R, the Cas9 protein and the sgRNA in specific ratios. We have learned that it is beneficial to use extra guide RNA in order to assure all of the Cas9 protein is loaded with guide strand. The preadipocytes are first grown to about 80-90% confluence, then trypsinized, counted, and centrifuged to achieve the cell pellet with the correct concentration of cells for the experiment. Once the cells are resuspended in the appropriate volume of buffer R, they are pipetted into a 10ul or 100ul Neon Tip. The tip is then placed into the Neon electroporation chamber and the electroporation is performed. There are several variables in the electroporation procedure that we have optimized for preadipocytes, including voltage, pulse, wavelength, cell number, concentration of Cas9 and sgRNA. Thus far we have identified conditions for both the 3T3 L1 mouse fibroblast (preadipocyte) cell line and primary mouse preadipocytes that yield high indel percentage rates. However, each new guide RNA may require modifications to these experimental variables and we are careful to fine tune the conditions for each sgRNA sequence.

Below in Figure 2 is representative data from analysis of an electroporation transfection using 10ul Neon tips with a Ncor1 sgRNA sequence. (Ncor1 has been reported to suppress metabolism and adipocyte "browning", similar to RIP140). **NOTE: We use the TIDE tool for analysis of indels for all of our experiments now, and this is extremely reliable and the standard in the field for analysis of CRISPR data *(Brinkman EK, et al., Nucleic Acids Research, 2014)*.** These data in Figure 2 show a 94% indel result under these conditions, a huge improvement over our previous methods and demonstration that a majority of cells will show ablated expression of the Ncor1 gene. We have obtained similar data for RIP140sgRNA sequences (see below) and for TGFbR (another gene that suppresses metabolism like RIP140m, see below), with indels of up to 98% and consistently over 90%.

Figure 2A.

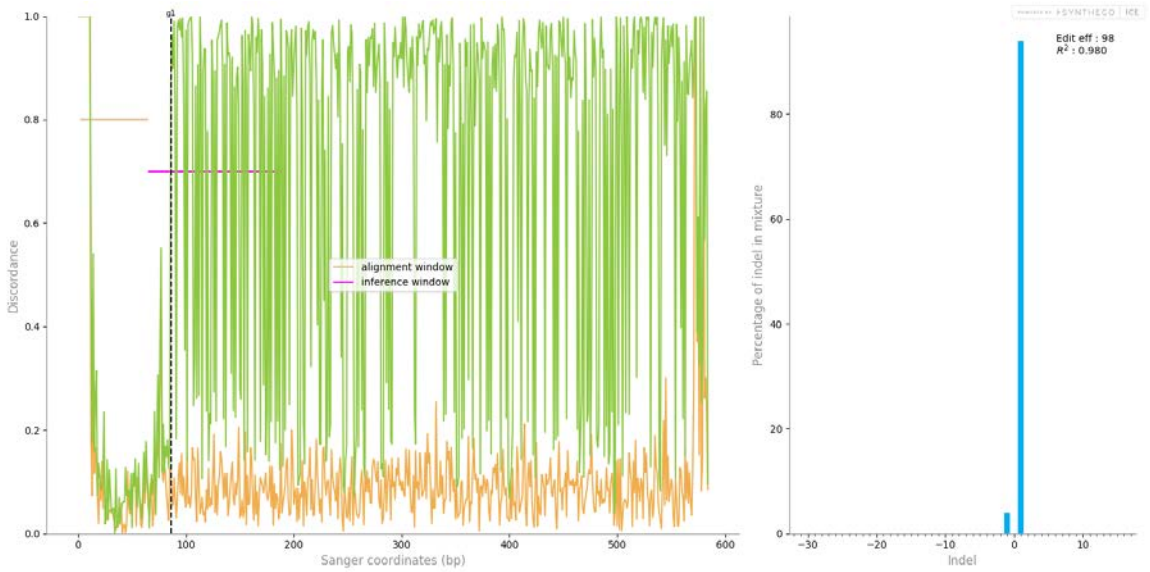

Figure 2B.

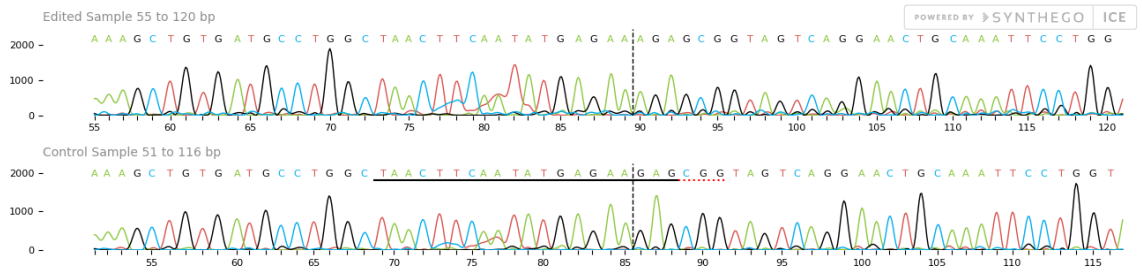

Figure 2. Effectiveness of electroporation on CRISPR-based gene editing with Ncor1 sgRNA. *A) 3T3-L1 fibroblasts transfected with PNA Bio SpCas9 sgRNA-Ncor1-674 by 10ul Neon Tips. DNA was extracted 72hrs post transfection and amplified with PCR primers designed to amplify a 700bp fragment spanning the gene edited portion of DNA. PCR products were purified with a column and sequenced with a gene-specific primer. Results of Sanger sequencing were uploaded to the Insertion/Deletion decomposition program designed by Synthego, called* ***Inference of CRISPR Editing (ICE)****[3] https://ice.synthego.com. **left panel** represents the discordance in the control sequence versus the knock out sequence, while the **right panel** represents the percentage of insertions or deletions (indels) detected (y axis), and the frequency in each nucleotide position around the cut site (x axis). B) demonstrates the trace file segments spanning the cut site generated by the ICE software. The guide sequence is underlined in the control trace on the bottom, while the PAM sequence is denoted by the red dotted line. Vertical dotted lines denote the expected cut sites in both the control and the edited traces. Data shown demonstrates an insertion of an additional A,* ***resulting in a 94% indel with a +1 frame shift, and a 4% indel with a -1 frame shift. Thus, a high majority of cells will be ablated in their expression of NCOR1.***

Comparison of commercially available SpCas9 from PNA Bio versus SpCas9 produced by Scot Wolfe's Lab through analysis of Inference of CRISPR Editing (ICE) produced with the larger Neon 100ul tips was also performed (Figure 3 below). These NEON tips allow gene expression KO in a much larger batch of cells, which will be suitable for future experiments on adipocyte implantation.

Figure 3A.

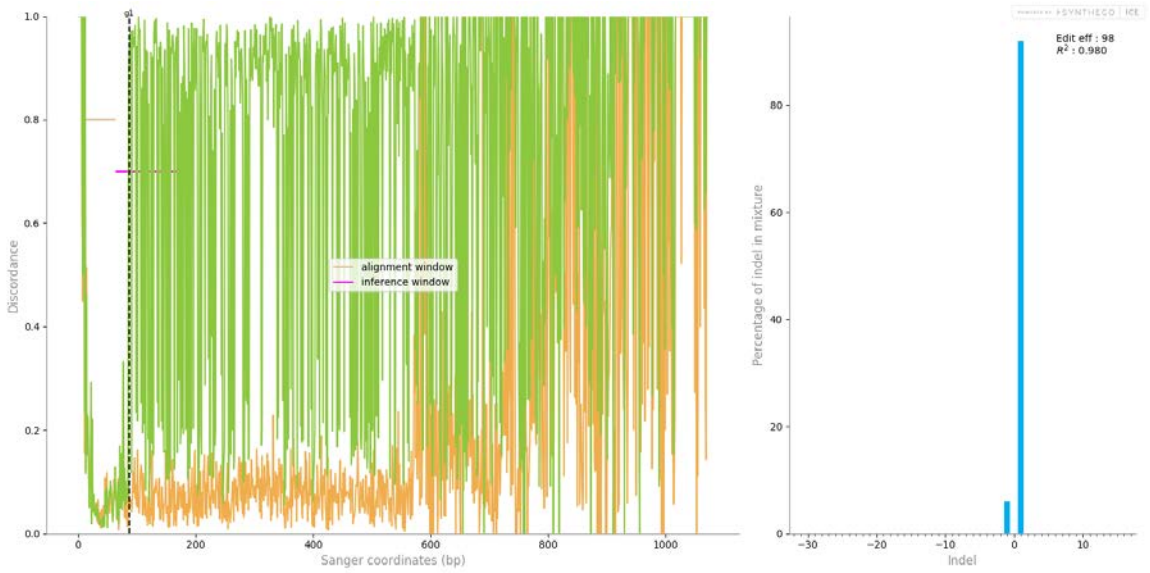

Figure 3B.

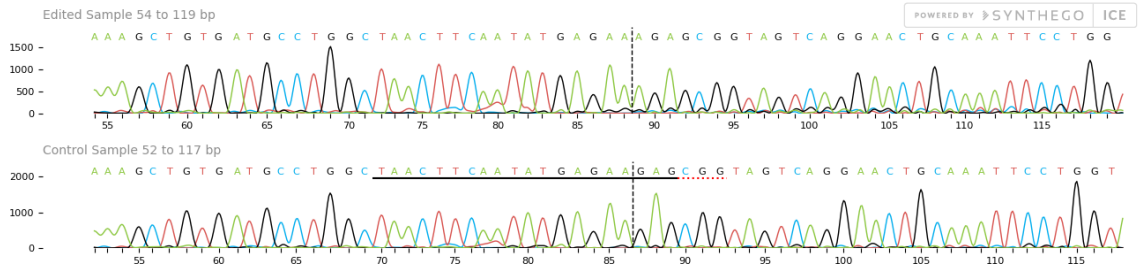

Figure 3C.

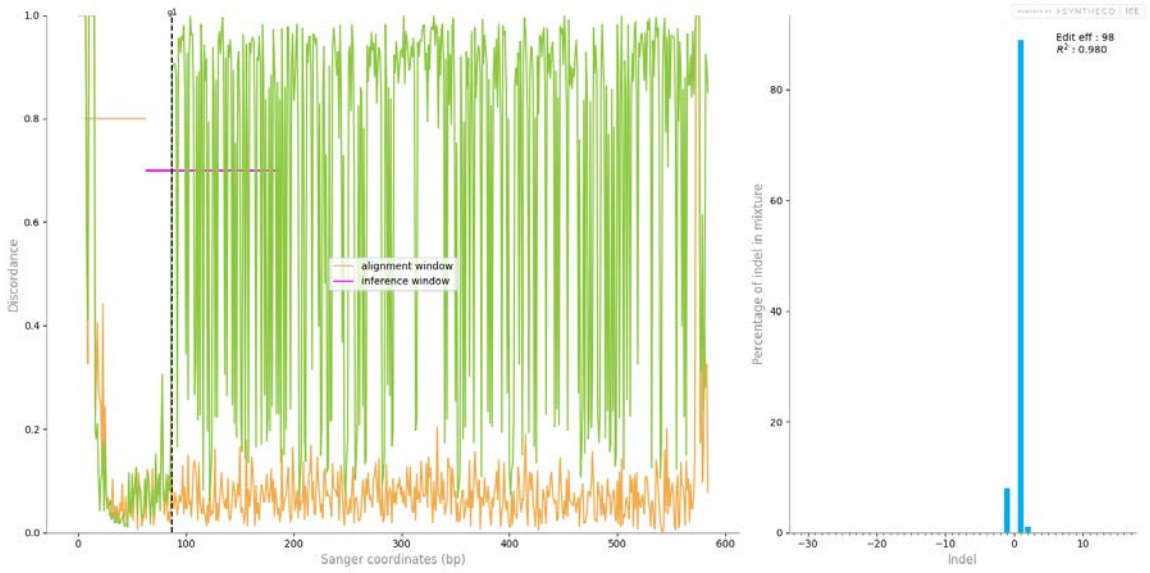

Figure 3D.

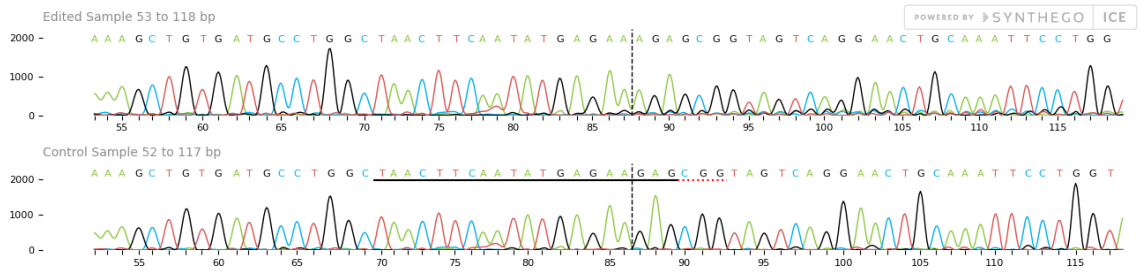

Figure 3. Comparison of SpCas9 sources to determine if there are significant differences between the commercial SpCas9 from PNA Bio (panels 3A and 3B) versus the spCas9 made by Scot Wolfe's lab (panels C and D) in the electroporation protocol. *3T3-L1 fibroblasts were transfected with PNA Bio SpCas9 or Scot Wolfe SpCas9 and sgRNA-Ncor1-674 by 100ul Neon Tips. DNA was extracted 72hrs post transfection and amplified with PCR primers designed to amplify 700bp fragment spanning the gene edited portion of DNA. PCR products were purified with a column and submitted with a gene-specific primer to GeneWiz for Sanger sequencing. Results of Sanger sequencing were uploaded to the Insertion/Deletion decomposition program designed by Synthego, called Inference of CRISPR Editing (ICE)[3] https://ice.synthego.com.* ***Panels A and C*** *left represent the discordance in the control sequence versus the knock out sequence. The right panels of A and C represent the percentage of insertions or deletions (indels) detected (y axis), and the frequency in each nucleotide position around the cut site (x axis).* ***Panels B and D*** *demonstrate the trace file segments spanning the cut site generated by the ICE software. The guide sequence is underlined in the control trace on the bottom, while the PAM sequence is denoted by the red dotted line. Vertical dotted lines denote the expected cut sites in both the control and the edited traces.*

The data in Figure 3 is summarized in Table 3 below. These data show equal indel analysis of 98% for each of the preparations of Cas9, allowing us to use Scot Wolfe's method of Cas9 preparation at the less expensive cost compared to purchasing commercially.

Table 3.

Sample	ICE indel score	KO-Score	ICE d	R Squared	Mean Discord Before	Mean Discord After	Guide Sequence
Ncor1-674 PNA Bio Cas9	98	98	86	0.98	0.161	0.751	TAACTTCAATATGAGAAGAG
Ncor1-674 Scot Wolfe Cas9	98	98	87	0.98	0.213	0.711	TAACTTCAATATGAGAAGAG

Table 3. Summary of the results for the comparison of PNA Bio SpCas9 versus Scot Wolfe Lab SpCas9. Ice Indel score is the percentage of editing efficiency as determined by comparing the edited trace sequence to the control trace sequence. The KO score represents the proportion of cells that have either a frameshift-inducing indel or a large indel in a protein-coding region (21bp or greater in length), which are likely to generate a complete loss-of-function mutation. ICE d (ICE discordance) is an algorithm used to detect complex or unexpected edits such as large insertions or deletions. R squared is the model fit score. When ICE linear regression is calculated the Pearson correlation coefficient (r) is also calculated. The higher the R squared value indicates the more confident you can be in the ICE score.

Analysis of TGFbR gene deletion using 4 different sgRNAs and the electroporation method (Tables 4 and 5 below). Table 4 shows a representative set of data from screening several sgRNAs against the TGFbR showing variability in their effectiveness, in which we identify one sequence against a segment within exon 4 with very high efficiency (97%). A key issue is whether the extent of gene modification (indels) survives multiple rounds of cell division, as one might imagine that the KO cells may not grow as fast as cells that were not modified. As shown in **Table 5**, this is not the case, as cells that were expanded in plates after electroporation for 7 days show the same level of indels as cells that were assayed only 72 hours after transfection. We have assured in other experiments not shown here that this is also the case for RIP140 deletion, i.e., continued high levels of gene KO over many generations of cell divisions.

Table 4. Candidate sgRNAs for TGFβR KO screening in 3T3-L1 cells

sgRNA	Sequence	Efficiency	Consistency
TGFbR-2 (exon 4)	AGAGCGTTCATGGTTCCGAG	97-97.6%	yes
TGFbR-4 (exon 2)	AATTATCCTTTGTACAGAGG	47.1-50%	no
TGFbR-6 (exon 2)	TTTCTGCCACCTCTGTACAA	75-79.5%	yes
TGFbR-7 (exon 2)	GCAAAGACCATCTGTCTCAC	81-84%	yes

Table 5. Time course of gene deletion efficiency assayed by TIDE

Condition	Surface replated	72 hours post Tx	7 Days post Tx
TGFβR-6 KO3	12w/p →12w/p	94.5-93.7%	96.0 – 94.5%
TGFβR-6 KO4	12w/p →6w/p	92.1-91.3%	92.4 – 89.4%

w/p is used to denote "well plate"

High reproducibility of indel efficiency using the electroporation method has been established in this project. Once a screen identifies a high efficiency sgRNA sequence, it is critical that it retains high reproducibility among different electroporation batches. We tested reproducibility of a high efficiency sgRNA we identified in a screen of sgRNAs against exon 4 regions of the RIP140 gene. In three separate electroporation experiments with three separate pools of preadipocytes we observed remarkable reproducibility of the indel generation percent: 95% in all three of the biological replicates. **This data is presented in Figure 4 below.**

Figure 4. RIP140 KO gene editing in primary preadipocytes – candidate sgRNA IDT2 (95% efficiency, 84% frameshift)

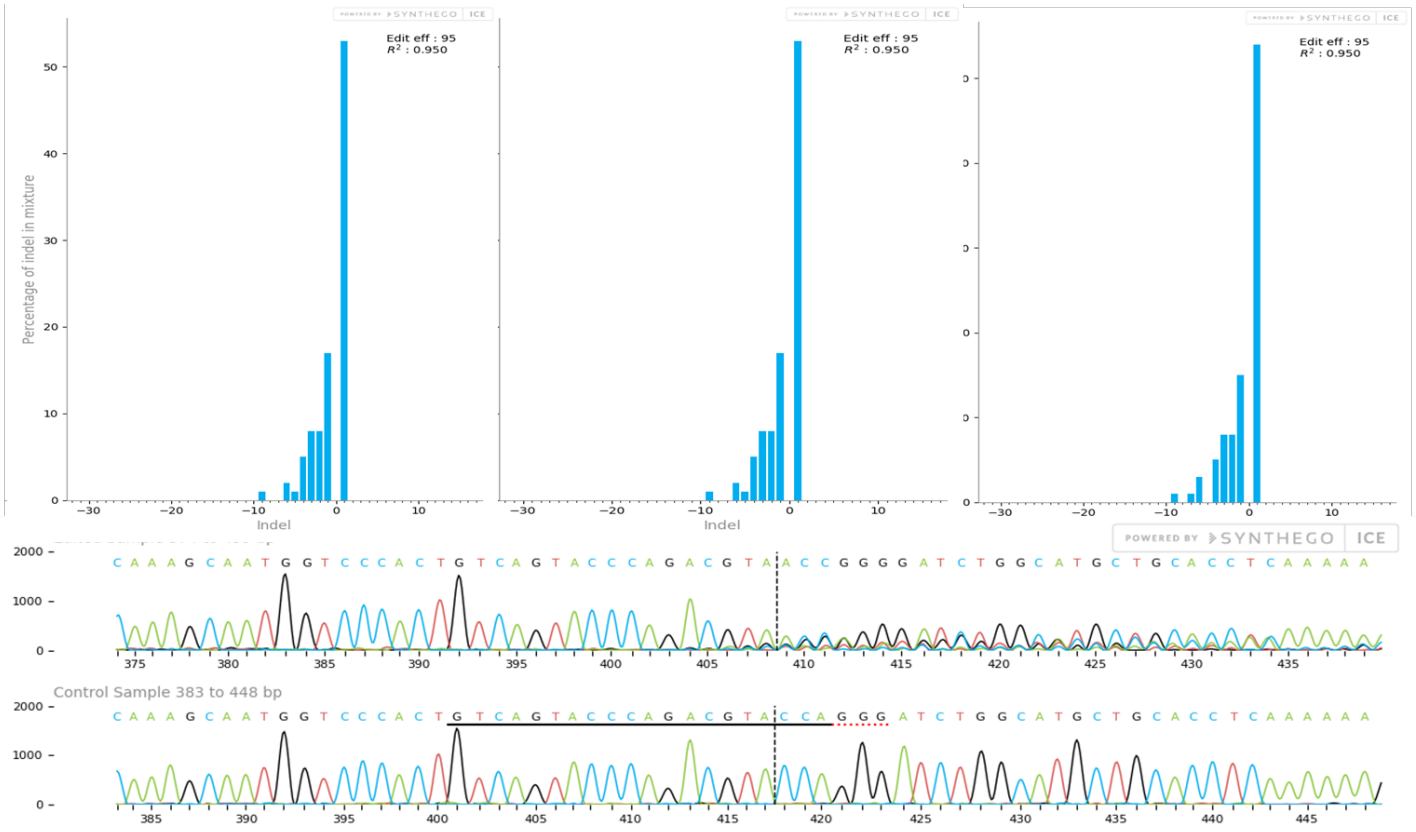

Figure 4. RIP140 indels induced by RIP2140 sgRNA denoted as IDT2 against a region of exon 4. *Three separate pools of preadipocytes were electroporated in different experiments to determine reproducibility of indel generation efficiency. Conditions and analysis by TIDE are similar to those described in the legend for Figure 3 except that primary mouse preadipocytes were used rather than 3T3-L1 cells.*

RIP140 KO in preadipocytes by electroporation of Cas9/sgRNA does not inhibit their subsequent differentiation into adipocytes. A crucial step in our overall strategy is that the method of CRISPR-mediated gene deletion AND the KO of the target gene itself are not deleterious to the

ability of preadipocytes to differentiate into adipocytes. This is important because our ultimate intention is to implant the adipocytes, not preadipocytes, into mice for therapeutic effects. This is based on preliminary data by the Corvera lab that showed preadipocytes do not form large fat pads when implanted into mice, as adipocytes do. Therefore, we tested the ability of preadipocytes that had been electroporated with RIP140 sgRNA to differentiate **(Figure 5).** While there is some variability in differentiation percentage, with high plating density we observe virtually full differentiation efficiency (see well denoted RIP140 sgRNA IDT3 KO4 in **Figure 5).** These important data indicate that we have successfully developed methods for permanent KO of RIP140 (and other genes) in preadipocytes, while the preadipocyte cells retain their ability for high efficiency differentiation to RIP140-deficient adipocytes.

Day 6 post differentiation

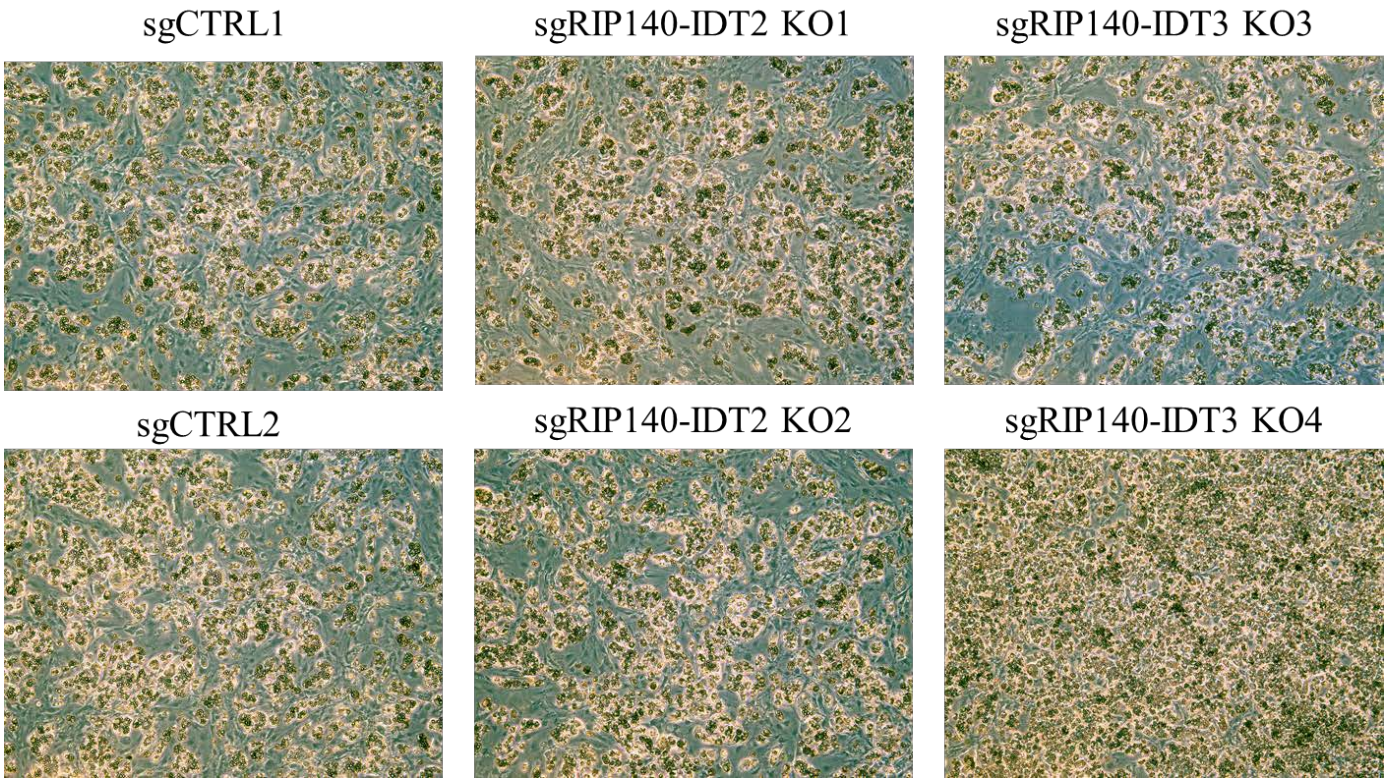

Figure 5. High efficiency differentiation of electroporated preadipocytes deficient in RIP140. *Shown are different wells of preadipocytes that were treated with either scrambled sgRNA (sgCTRL) or RIP140 sgRNA IDT2 or IDT3 (all with Cas9 protein), and then exposed to differentiation media. Significant differentiation is observed in all cases. We have also found that when the electroporated preadipocytes are plated at high density, a higher per cent of differentiation is observed and consistently obtained (similar to well RIP140 KO4 in the Figure).*

Upregulation of thermogenic UCP1 in adipocytes deficient in RIP140 through CRISPR-mediated indel generation. A key endpoint of CRISPR-mediated gene KO in adipocytes is upregulation of energy expenditure as reflected in increased expression of the mitochondrial uncoupling protein UCP1. We have reached that milestone with one of our RIP140 sgRNA sequences—RIP140 sgRNA IDT3. As shown in **Figure 6,** RIP140 deletion with this Cas9/sgRNA combination elicited a 10 fold increase in UCP1 mRNA expression. It is not clear why the RIP140 sgRNA IDT2 did not achieve this result, and we are investigating that sequence for the reason. It is possible that enough intact or partially active mRNA for RIP140 remains in these cells, and we will be able to test that. Importantly, we can upregulate UCP1 by an order of magnitude in RIP140 sgRNA IDT3-treated cells even in the absence of cAMP stimulation by adrenergic agonists. We are in the process of characterizing this effect in much more detail (related to oxygen consumption, UCP1 protein levels, and secreted protein expression), and these results will be reported in the next progress report.

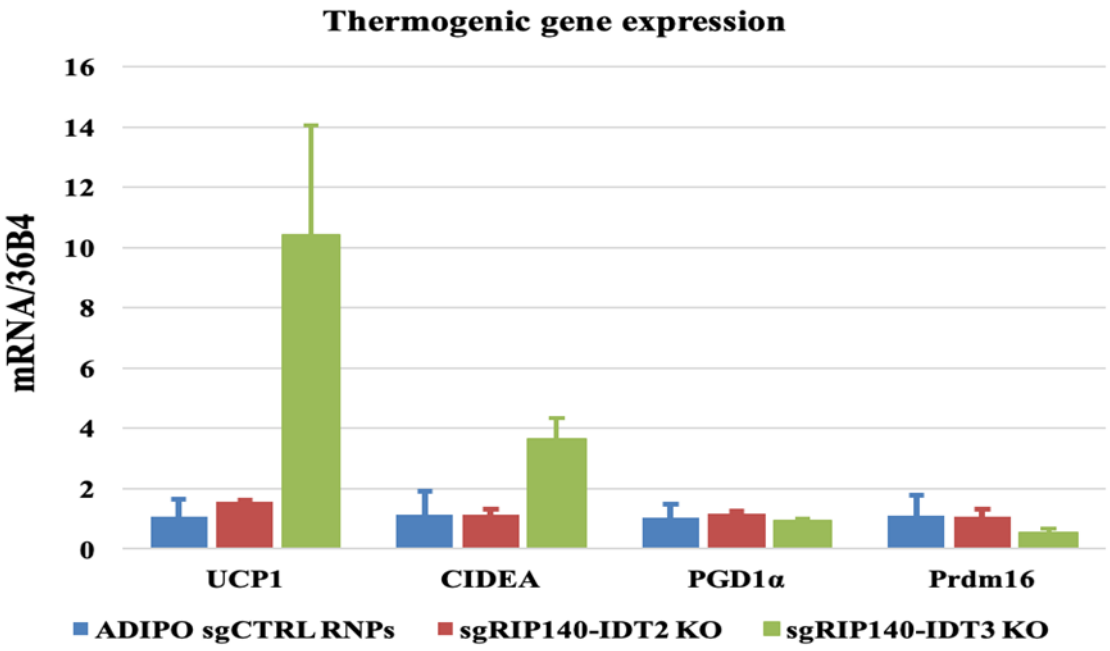

Figure 6. Upregulation of UCP1 expression by KO of RIP140 in adipocytes. *Preadipocytes were electroporated with RIP140 sgRNA IDT3 to delete RIP140, and then differentiated into adipocytes. The 4 genes denoted were then assayed for expression by RT-PCR. Two thermogenic genes, UCP1 and Cidea, were significantly increased in expression by RIP140 deletion. Our expectation is that in follow-up experiments that test the responses to adrenergic agonists, we will find even greater increases in expression of thermogenic genes upon RIP140 KO based on previous experience.*

The above experiments were performed within the Czech lab (site 1), but with close association and collaboration with the Corvera group (site 2). This joint effort is optimal for the project since we are also in the process of transferring this technology to the human adipocytes that are generated in the Corvera lab. We have jointly identified human RIP140 sequences for sgRNAs that are being used on human preadipocytes (**see Table 2 above**) and are in the process of screening these for the best indel generation and RIP140 KO effects. We anticipate that we shall soon be at the same stage with human adipocytes as we have defined for the mouse adipocytes in the experiments presented here. Those experiments on human adipocytes will be completed in the next few months and will be presented in the next semi-annual progress report.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We plan to complete the testing and characterization of gene deletions of RIP140, TGFbR and NCOR1 in mouse adipocytes and human adipocytes to assure we have the optimum CRISPR-mediated gene deletion conditions for enhancing energy expenditure, fatty acid oxidation and beneficial factor secretion. We then anticipate initiating the adipocyte implantation experiments into mice to assess potential beneficial metabolic effects this next project period, as proposed in our original SOW.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

New method for transfecting preadipocytes with Cas9/sgRNA is described in this report, with potential for patent filing. This technology has not yet been shared with the scientific community or the public.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*

- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Silvia Corvera – no change
Tiffany DeSouza – no change

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

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

The Following Grants have been funded since the last reporting:
Title: **Adipocyte to neuron signaling in thermogenic programing of white adipose DK116056**
Effort: 1.20 calendar months
National Institutes of Health (NIH)
Performance Period: 3/7/19-1/31/24
Level of Funding:

Title: **Long Non-coding RNA as Mediators of Metabolic Disease (Freedman)**
Effort: 0.60 calendar months
NIH-National Heart, Lung, and Blood Institute
Performance 2/1/19-1/31/23
Level Funding

The Following Grants have ended since the last reporting:
Title: Inter and intra-cellular trafficking pathways for natural and therapeutic RNAs/R01GM108803 (Khvorova)
Effort: 1.20 calendar months
National Institutes of Health (NIH/NIGMS)
Performance Period: 8/15/14-7/31/18
Level Funding:

Title: **University of Massachusetts Center for Clinical and Translational Science (UMCCTS)/UL1** TR001453, Luzuriaga (PI)/TL1 TR001454, Corvera (PI)
Effort: 0.60 calendar months
National Institutes of Health (NIH/NCATS)
Performance Period: 8/14/15-3/31/19
Level Funding

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

PR170457P1: CRISPR-Based Gene Editing to Induce Thermogenic Adipose Tissue in Type 2 Diabetes

PI: Silvia Corvera Univ. Massachsetts Medical School, MA Budget: $1,015,617.00

Topic Area: Type 2 Diabetes Mechanism: PRMRP/IIRA CDMRP Grant

Research Area(s): 0800 Award Status: 15 July-2018 to 14-July-2021

Study Goals:

- Improve CRISPR delivery efficiency to preadipocytes and adipocytes for gene deletion.
- Develop Nrip1 ablation in mouse & human preadipocytes with high efficiency, and test other genes
- Implant genetically modified mouse & human adipocytes into mice and "humanized" mice.
- Test CRISPR delivery technology directly in adipose depots in mice

Specific Aims:

Aim 1. We shall optimize and define the most potent sgRNA sequences for maximal efficiency of RIP140 deletion by screening dozens of sgRNA sequences against RIP140 in primary mouse adipocytes and in human "beige" adipocytes *in vitro*.

Aim 2. We shall test the hypothesis that primary mouse and human adipocytes deleted of RIP140 by our Cas9/sgRNA nanoparticles (Crips) *in vitro* mediate markedly enhanced improvements in glucose tolerance and insulin sensitivity upon implantation into obese mice and obese "humanized" mice, respectively.

Aim3. We will test the hypothesis that Crips can be directly injected into adipose depots in mice and in "humanized" mice (with implanted human adipocytes) in order to delete RIP140, enhance "browning" and improve glucose tolerance.

Key Accomplishments and Outcomes:

- Major Advancement: efficiency of Cas9/sgRNA-based gene deletion to over 90% in preadipocytes

Publications: none to date

Patents: none to date

Funding Obtained: none to date