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14. ABSTRACT:
Myelodysplastic Syndromes (MDS) are a group of clonal hematopoietic disorders characterized by bone marrow failure and risk of progression to Acute Myeloid Leukemia (AML) in approximately 30 percent of the cases. Aberrant DNA methylation is considered a dominant mechanism for Tumor Suppressor Genes silencing during MDS evolution to AML, but the causes leading to aberrant DNA methylation remain elusive. This proposal builds on our recent discovery of a novel class of RNAs, the DiRs or **D**NMT1-**i**nteracting **R**NAs, involved in cell type-specific DNA methylation patterns. Based on these findings, we hypothesize that DNA methylation changes can be corrected by RNAs. We aim to demonstrate that: a) by inducing transcription within targeted methylated genomic loci or b) by utilizing oligonucleotides mimicking the function of DiRs and able to specifically target methylated loci, we will be able to reduce level of methylation and consequently rescue the expression of the respective silent gene. In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

15. SUBJECT TERMS

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1. INTRODUCTION:

Myelodysplastic Syndromes (MDS) are a group of clonal hematopoietic disorders characterized by bone marrow failure and risk of progression to Acute Myeloid Leukemia (AML) in approximately 30 percent of the cases. Aberrant DNA methylation is considered a dominant mechanism for Tumor Suppressor Genes silencing during MDS evolution to AML, but the causes leading to aberrant DNA methylation remain elusive. This proposal builds on our recent discovery of a novel class of RNAs, the DiRs or **D**NMT1-**i**nteracting **R**NAs, involved in cell type-specific DNA methylation patterns. We have found that DNMT1 binds to RNA with stronger affinity than DNA of the same primary structure. This interaction inhibits DNMT1 enzymatic activity thereby preventing DNA methylation and the resultant silencing of the corresponding DiR-regulated gene loci. Based on these findings, we hypothesize that DNA methylation changes can be corrected by RNAs. We aim to demonstrate that: a) by inducing transcription within targeted methylated genomic loci or b) by utilizing oligonucleotides mimicking the function of DiRs and able to specifically target methylated loci, we will be able to reduce level of methylation and consequently rescue the expression of the respective silent gene. In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), an important gene exploiting not only cell-cycle regulator functions, but revealing specific features in the regulation of hematopoietic progenitor cell fate. *P15* is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Myelodysplastic syndrome; p15, DNA methylation; RNA

What were the major goals of the project?

In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), an important gene exploiting not only cell-cycle regulator functions, but revealing specific features in the regulation of hematopoietic progenitor cell fate. *P15* is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR*

What was accomplished under these goals?

Aim 1. *To reduce P15 locus specific genomic methylation by induction of its respective DiR*

Our previous studies demonstrated that downregulation of *ecCEBPA* led to decreased CEBPA mRNA and increased DNA methylation levels, whereas ectopic expression of *ecCEBPA* resulted in an opposite outcome. Further, we demonstrated that RNA has a stronger affinity than DNA to DNMT1 and that RNA specifically interacts with the DNMT1 catalytic domain, leading to the hypothesis that RNA oligonucleotides could be utilized as gene specific demethylating agents. We continued exploring this avenue and chose as a model for this study two tumor suppressor genes frequently methylated in cancer: the *CDKN2A* (aka *P16*) and *CDKN2B* (aka: *P15*). While *P16* is commonly methylated in solid tumors, *P15* is silenced in myeloid disorders. Therefore, we decided to apply the same RNA-mediated demethylating approach to both genes. During the third funded year, we have finalized the conditions to perform RNA- and DNA- Fluorescence In Situ Hybridization (FISH) for *P15*. Given that both techniques do not require a large number of cells, we will be able to apply this procedure on MDS primary samples, not only to assess the integrity of the locus, which is often deleted in cancer, but also to visualize the presence of *P15* DiRs and their induction and/or upregulation upon DiR-mimicking oligonucleotides delivery. For DNA Fluorescence in situ Hybridization (DNA-FISH) we used BAC probes distinguishing local or full deletion of the DNA (**Figure 1a**). BAC Clone 149I2 covered both *P15* and *P16* loci, while 454D15 covered only the *P16* locus. *P16* maps telomeric to *P15*. Positive hybridization of the Chromosome 9 control probe indicated a successful hybridization. By this approach we confirmed the presence of the *P15* locus in KG1a and the

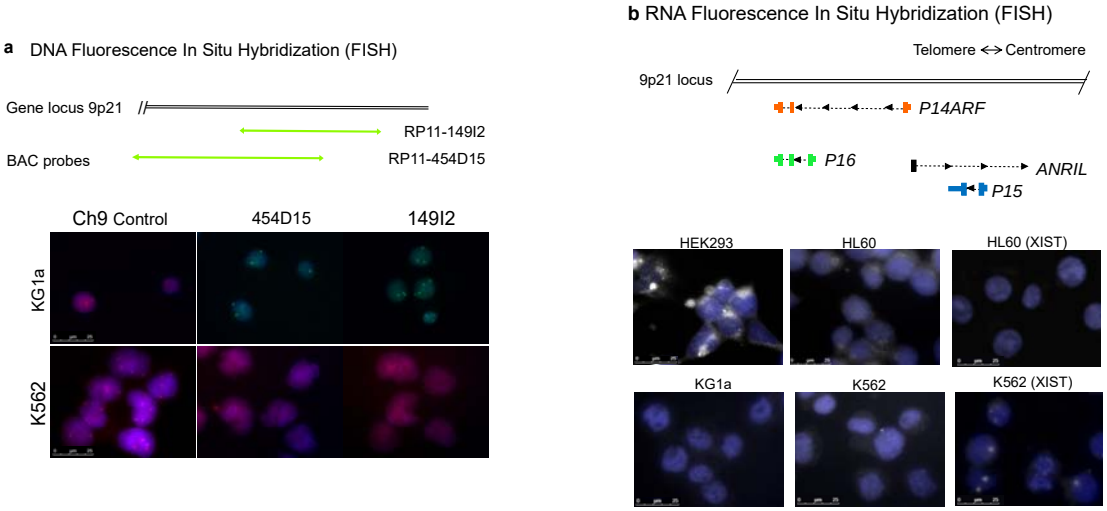

Figure 1. *P15* visualization by DNA and RNA FISH. a, Upper Panel: Schematic of BAC probes used for DNA FISH of *P15-P16* gene loci, in KG1a and the K562 lines. **Lower Panel:** DNA-FISH for *P15* and *P16* gene loci in KG1a and K562 lines; **b, Upper Panel**: Diagram of coding and noncoding transcripts on the *P15* /*P16* locus (Taken from UCSC Genome Browser); **Lower Right Panel:** RNA FISH of *P15* in HEK293 (high), HL60 (low), KG1a (not expressing) cell lines and K562 (deleted for the locus).

absence of the entire *P15*/*P16* in K562. The *P15-16* locus is indeed frequently deleted in cancers.

Single molecule RNA FISH is a methodology for detecting and localizing particular RNA. Stellaris RNA FISH technology is based on the use of fluorescently labeled tiling oligonucleotides complementary to a sequence of interest (Raj, Nature Methods 2008). Unlike DNA FISH, RNA FISH is strand-specific, enabling the visualization of sense and antisense RNA strands. The use of tiling probes also has the added benefit of increasing the chance of hybridization success within the oligo pool and reducing the effect of nonspecific hybridization as multiple co-localizing probe signals are needed to visualize a single RNA molecule. The *P15* probe set was designed against nucleotides 1-516, 517-1650, 1651-2198 of NM_004936.3 covering the coding sequences only, and detects both *P15* variants, *P15* NM_004936.3 and NM_078487.2 (www.biosearchtech.com). Visualization of RNAs was carried out following the Modified Fluorescent In Situ Hybridization protocol (Stellaris, BioSearch Technologies). The Ready Designed XIST probe set was used for control of nuclear permeabilization and compartment-specific hybridization. All solutions were supplemented with vanadyl complex RNAse inhibitor (New England Biolabs). RNA FISH images was generated from a composite of z-stack images. Raw images were acquired on a Leica DM 5500B Microscope with a 100W high-pressure mercury lamp. Autofluorescence was checked for by simultaneous acquisition of images in the opposite filter channel. Images were taken with a Leica DFC 350 FX camera (CCD with a Peltier cooling system). 10 µM of z-stack images were acquired in 0.3 mM steps. Images were assembled and contrast-enhanced using FIJI (FIJI is Just Image J; https://fiji.sc/) as per manufacturer's recommendations (**Figure 1b**).

Using a Click-iT technology combined with deep sequencing of nuclear RNAs we have identified transcripts with features similar to *ecCEBPA* (enriched in the nucleus and transcribed during the S-phase), including those corresponding to the *P15* and *P16* loci. Click-iT® technology is based on the biorthogonal click chemistry reaction, which enables the metabolic incorporation of ethynyl uridine (EU), a "clickable" ribonucleoside, into RNA during nascent RNA synthesis. Biotin is "clicked" onto the nascent chain and streptavidin magnetic beads capture all newly synthesized transcripts. To compare the transcriptional profiles under these conditions with our previous results, we performed RNA-seq on total and nuclear RNA fractions of unsynchronized and S-phase synchronized HL-60 cells. Although HL-60 lacks both *P16*/*P15* proteins, the entire locus display antisense transcription of *ANRIL*, a long non-coding RNA antisense to *P15* reported as a *P15* silencer. *ANRIL* acquires the highest levels of expression during the S-phase in the nuclear compartment, pointing to a potential counteracting effect on the DiR-like transcripts arising from the *P15* locus. To assess the minimum number of transcripts driving the locus demethylation, we have generated an array of standard curves to measure both gene reactivation and DiR-like transcripts, based on copy number quantitation. This strategy allows an accurate quantification of the transcriptional activity not only on the bulk cell population, but also at single cell resolution.

We have started designing a mapping strategy including 5'3' RACE, northern blot analysis and RNAse protection assay to identify DiR-like transcripts in both expressing and not-expressing cell lines (**Figure 2**).

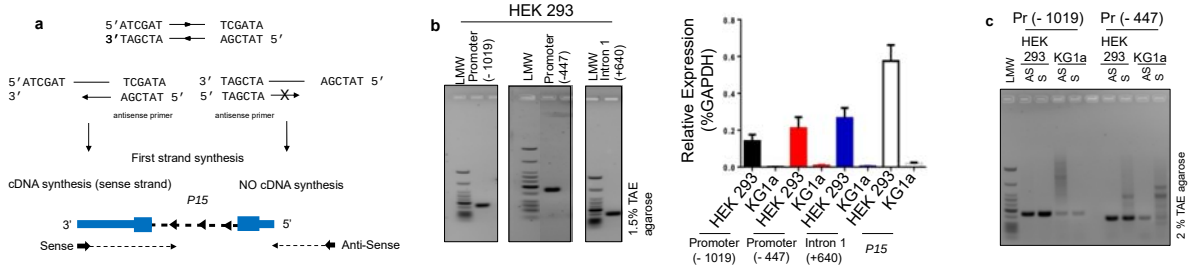

Figure 2. Identification of Sense(S) and Anti-Sense (AS) RNA using a ssPCR RT-PCR approach in *P15* locus. a, Schematic of cDNA synthesis for S/AS using different primers spanning the entire *P15* locus; **b**, Amplification of sense transcripts within promoter and intron 1 of *P15* locus by end point PCR on *P15* expressing cell line HEK293 (left panel) and quantitative RT-PCR on HEK and KG1A (not-expressing cell line) (right panel); **c** Amplification of sense and antisense transcripts within the distal promoter.

These results suggest a complex regulation of the locus that can be dictated by a fine-tuning of sense

and antisense transcription and enhance the relevance of RNA as a therapeutic tool to control gene expression.

Aim 2. *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

The goal of this aim is to evaluate whether RNA can be repurposed as a gene-specific demethylating tool to correct aberrant DNA methylation. P15 is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. This past year, our laboratory has been testing a double stranded RNA platform, the short activating RNAs (saRNAs) to reactivate *P15* gene expression. saRNAs are small double stranded RNAs that were shown to induce gene expression in a gene-specific manner, yet the mechanism behind this reactivation remains unknown. Our hypothesis is that the saRNAs might be acting as DiRs-mimicking molecules, and we will investigate whether saRNAs induce demethylation of targeted loci. Thus, in collaboration with the U.K. biotech firm MiNA Therapeutics (for further information please refer to the OUTREACH Study: https://clinicaltrials.gov /ct2/show/NCT02716012), three saRNAs targeting different genomic locations within the *P15* locus were designed: saP15-313 (promoter); saP15-11 (first- exon); and saP15-56 (intron).

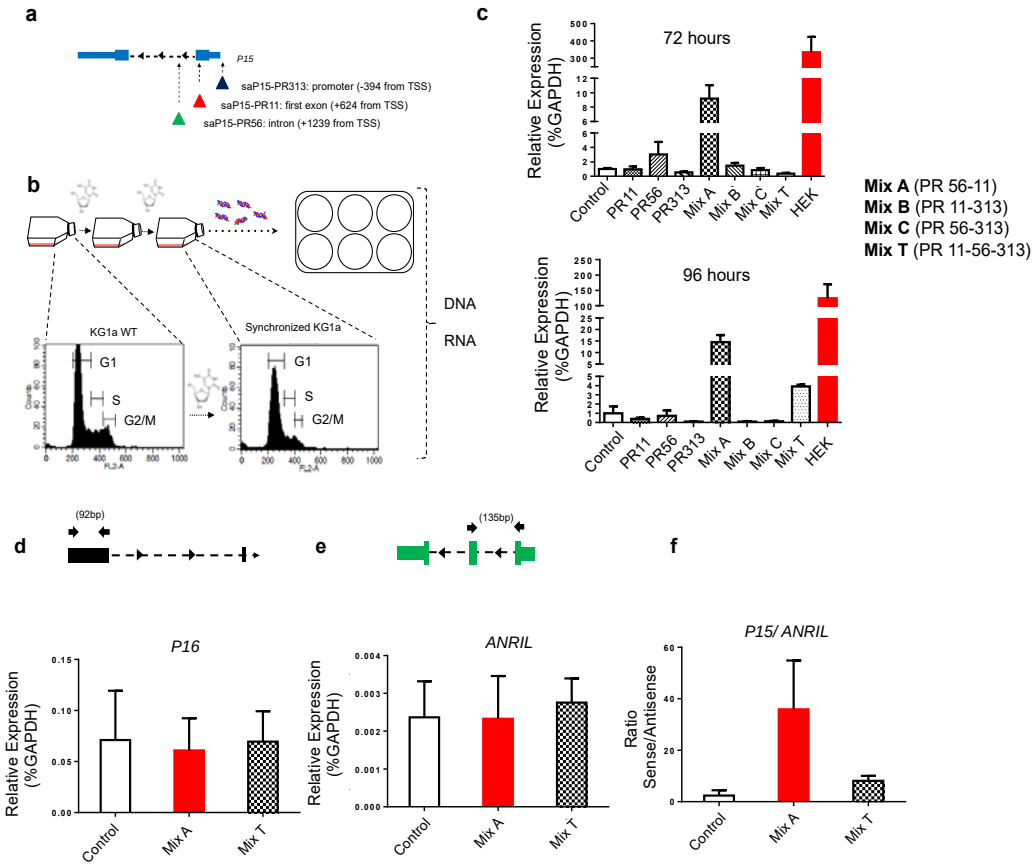

Figure 3. *P15* re-activation upon 50 nM saRNA-treatment. a, Schematic of saRNAs targets on to *P15* locus. **b**, The KG1a cell were synchronized at G1/S phase with double thymidine block (2,5 mM thymidine).1*10^6 cells were electroporated by nucleofection with 50 nM of saRNA. All possible saRNAs combinations were tested. DNA and RNA samples were collected at 72 and 96 hr post transfection to evaluate *P15* methylation profile and expression, respectively. **c**, Re-activation of *P15* gene expression 72 hours (upper right panel) and 96 hours (lower right panel) upon transfection with saRNAs Mix A and Mix T; **d**, diagram of primer location for *P16* (upper panel) and relative gene expression upon saRNAs (lower panel); **f**, diagram of primer location for *ANRIL* (**upper panel**) and relative RNA expression upon saRNAs (**lower panel**); **e**, ratio between Sense and *ANRIL*) upon saRNAs.

All combinations of the three saRNAs were delivered into the myeloid cell line KG1a, which is heavily methylated at the *P15* locus. Given DNMT1's activity peaks during the G1/early S-phase, we decided to synchronize the cells by double thymidine block before transfection as previously done (1).

Seventy-two and ninety-six hours post-transfection, RNA and DNA were collected to examine *P15* expression and *P15* locus DNA methylation changes upon saRNAs treatment, respectively. Strand-specific qRT-PCR results showed increase in *P15* expression with 50 nM saRNAs at seventy-two- and ninety-six-hours post-transfection with saP15-PR56, saP15-PR313, Mix (PR11-PR56) and Mix3 (**Figure 3 a-c**). saRNAs did not affect expression levels of the neighboring gene *P16* and overlapping antisense transcript *ANRIL* (96 hours post-transfection) (**Figure 3 d-e**). Reactivation of *P15* was associated with DNA methylation changes detected by Combined Bisulfite Restriction Analysis (COBRA) within the two CpG islands spanning the promoter and the first exon (between -280 and +802 bp from the transcription start site) and the second exon of *P15* locus as compared to the scramble control (FLUC) (**Figure 4**).

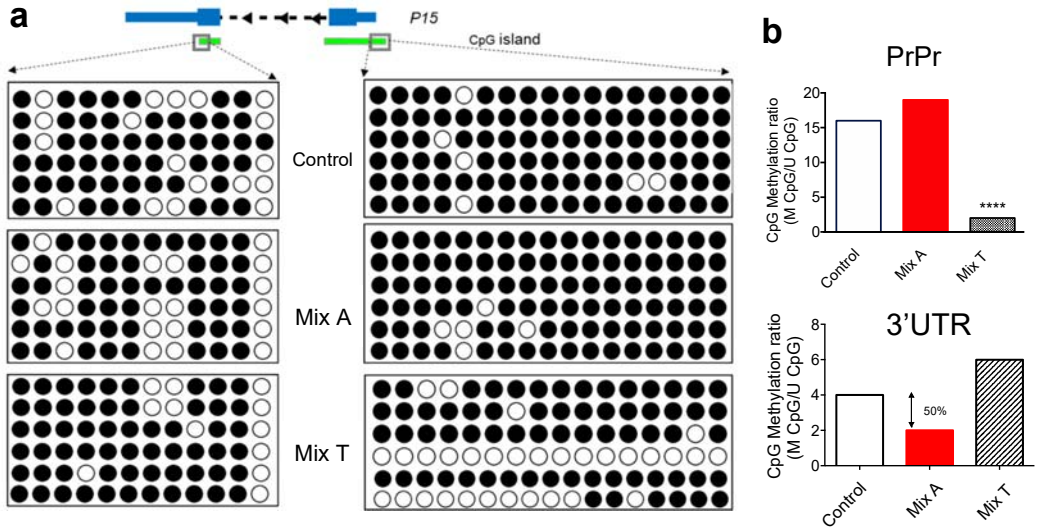

Figure 4. *P15* DNA methylation changes upon 50nM saRNA-treatment, 96 hours post-transfection. **a,** Lollipop plot showing DNA methylation profile of Proximal Promoter (PrPr) and 5'UTR of *P15* locus upon saRNAs treatment (bisulfite sequencing, BS); **b,** Histogram on the right upper panel shows the ratio between methylated (M) and unmethylated (UM) CpGs within the PrPr in saRNAs treated samples, 96 hours post transfection. Mix T induces more than 80% decrease of DNA methylation as compared to control. Histogram on the right lower panel shows the ratio between M and UM CpGs in saRNAs treated samples, 96 hours post transfection. 50% indicates the percentage of UM CpGs in MixA samples, as compared to control. Number of clones analyzed ≥ 6. M: methylated; UM: unmethylated; ●: methylated CpG; ○: unmethylated CpG.

We are currently completing the analyses of the remaining CpG islands encompassing the *P15* locus. Remarkably, 5-azacytidine (5AC), a clinically approved hypomethylating agent, did not induce changes in *P15* mRNA levels greater than those observed upon saRNAs treatment. Moreover, *P15* expression levels did not mirror the changes in DNA methylation for all the treated samples similarly to other studies (17). These results suggest the usage of saRNAs as therapeutic tools to target aberrantly methylated gene *loci* and to restore gene expression.

Further, we conceived a different strategy of gene-specific DNA demethylation, through a collaborative effort with the laboratory of Prof. Vittorio de Franciscis, Istituto di Endocrinologia ed Oncologia Sperimentale, Naples, Italy, by developing aptamers as inhibitors of DNMT1. Aptamers are short synthetic nucleic acids or peptides that are selected for specific binding to target of interest. They are a new class of molecules that represents high affinity ligands or antagonists targeting disease-associated proteins. Besides being cost-effective and relatively easy to manipulate, the small size of aptamers (6-30kD) allow them to access binding pockets that are usually inaccessible to macromolecules. They have low toxicity, immunogenicity, but are able to retain high affinity in target binding.

In order to identify aptamer sequences with high affinity specificity and stability, we have performed three rounds of protein SELEX (Systematic Evolution of Ligands by EXponential enrichment), using as a bait the RNA sequence binding DNMT1 (R5) as described in Di Ruscio *et al*. (**Figure 5**). Three regions of R5 were randomized by chemical synthesis in order to generate three separate sub-libraries each containing the template for the entire R5 sequence with one short 4-5 bases randomized region. The three sub-libraries were mixed at equimolar concentration and used as template to generate the 2'-Fluoro-Pyrimidines (2'F-Py) modified nuclease resistant RNA starting pool. Randomized RNA pools were then subjected to three to four rounds of protein SELEX. The final pool was cloned and three promising aptamer sequences (Ce9, Ce10, Ce49) with good DNMT1 binding and improved serum stability have been singled out. We are currently testing the selected aptamers in cell culture and in vitro assay on K562, a laboratory myeloid cell line methylated within the *CEBPA* promoter and not expressing the gene and designing aptamer that could target the *P15* locus to achieve targeted DNA demethylation (**Figure 6).**

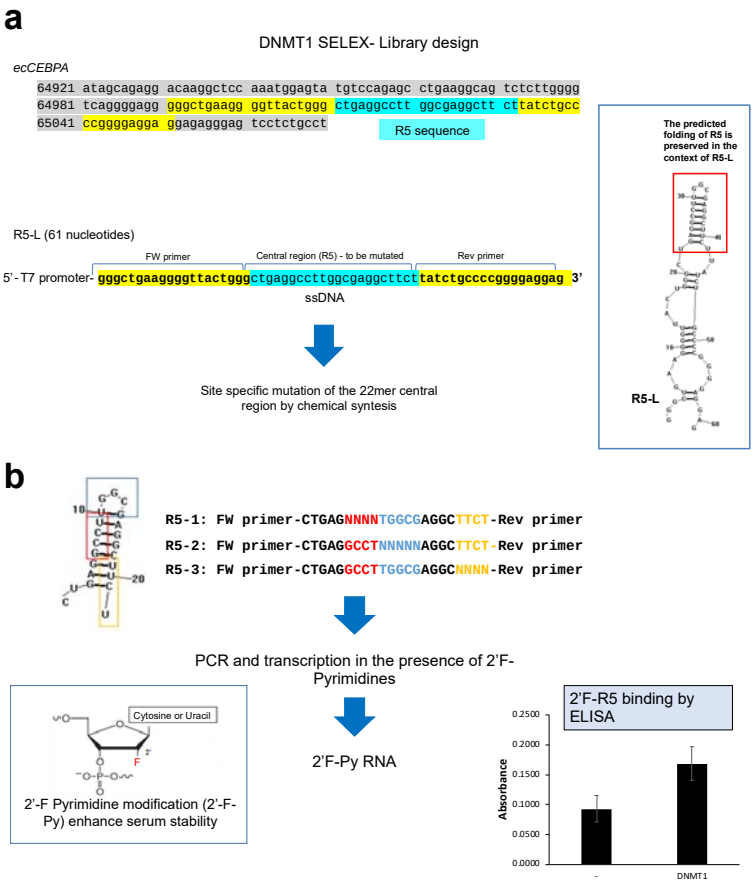

Figure 5. DNMT1 SELEX- Library design and approach. a, Three regions of R5 have been randomized by chemical synthesis to generate three separate sub-libraries (SL1, SL2 and SL3) each containing the template for entire R5 sequence with one short 4-5 bases (N) randomized region; **b**, The three sub-libraries have been mixed at equimolar concentration, used as template for the 2'F-Py modified RNA starting mixed pool, then subjected to three rounds of protein SELEX.

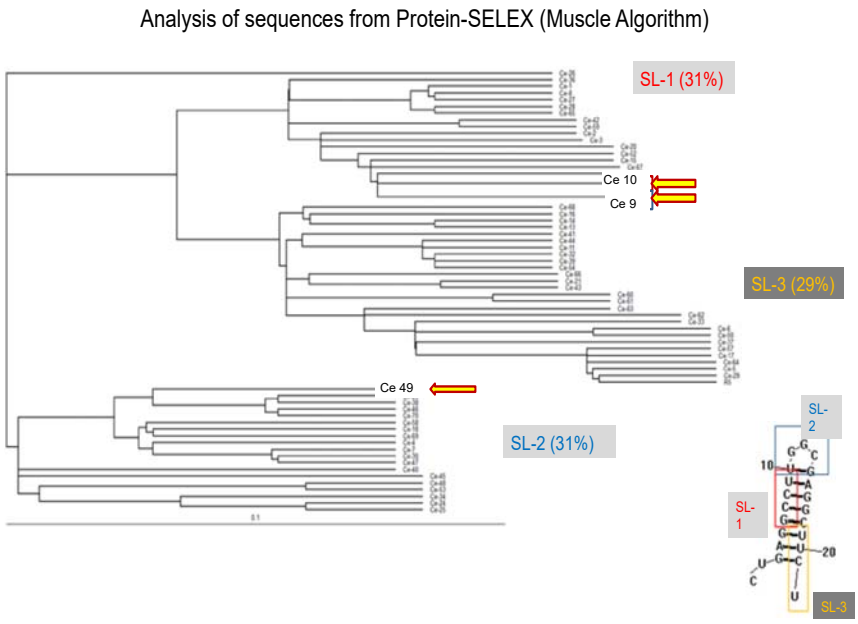

Figure 6. Selection of aptamers binding DNMT1 with stronger affinity than R5: Ce9-10-49

What opportunities for training and professional development has the project provided? Nothing to report

How were the results disseminated to communities of interest? Nothing to report

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

a) We will continue to characterize the DiRs in the *P15* and *P16* loci, including mapping their 5' and 3' ends, not only in cell lines but in primary MDS patient samples;
b) We will design and produce lentiviral particles for expression of specific *P15* and *P16* DiRs, and initiate experiments to express the first in cell lines, followed by expression in MDS patients samples in which these genes are not expressed in methylated;
c) We will assess the ability of these DiRs to induce demethylation and expression of *P15* and *P16*;
d) We will study the effect of short activating RNAs (saRNAs) on the *P16* locus;
e) We will continue to develop oligonucleotides which mimic the action of the DiRs, both triplex forming oligonucleotides (TFOs) and chimeric RNA oligonucleotides (CROs);
f) Finally, we are developing aptamers able to bind with high affinity DNMT1, but targeting specific genomic locations to activate tumor suppressor genes.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project? Nothing to report

What was the impact on other disciplines? Nothing to report

What was the impact on technology transfer? Nothing to report

What was the impact on society beyond science and technology? Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

Changes in approach and reasons for change: Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them: Nothing to report

Changes that had a significant impact on expenditures: Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to report

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:

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

  Journal publications.

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

  Other publications, conference papers and presentations..
- **Website(s) or other Internet site(s)**
- **Technologies or techniques**
- **Inventions, patent applications, and/or licenses**
- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project? No changes

Name:	Daniel G. Tenen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-6423-3888
Nearest person month worked:	2.4
Contribution to Project:	Dr. Tenen is overseeing the direction of the project
Funding Support:	This grant and other NIH grants

Name	Junyan Zhang
Project Role:	Senior Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Ms Zhang assists Dr. Tenen and other postdocs in this project
Funding Support:	This grant and other NIH grants

Name	Anais Wanet, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Dr. Wanet is focusing on Aim 1
Funding Support:	This grant and other NIH grants

Name	Mee Rie Sheen, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.77
Contribution to Project:	Dr. Sheen is focusing on Aim 2
Funding Support:	This grant and other NIH grants

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report

What other organizations were involved as partners? None

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable

QUAD CHARTS: Not Applicable

9. APPENDICES: None