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TITLE:	Single-Cell CRISPRa Screen to Identify Transcription Factors That Mediate Neuroblastoma Phenotypic Switching and Chemotherapy Drug Resistance
PRINCIPAL INVESTIGATOR:	Dr. Zhihui Liu
CONTRACTING ORGANIZATION:	The Geneva Foundation Tacoma, WA 98402
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Single-Cell CRISPRa Screen to Identify Transcription Factors That Mediate Neuroblastoma Phenotypic Switching and Chemotherapy Drug Resistance

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5d. PROJECT NUMBER

5e. TASK NUMBER

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Geneva Foundation
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Objectives: I hypothesize that there are other ADRN-and MES-specific TFs that can mediate trans-differentiation and in extension chemotherapy resistance. Furthermore, I propose that the combinatorial cooperation of several TFs will be more potent than a single TF to facilitate this process. To test my hypothesis, I propose two specific aims: 1) aim 1 is to identify TFs and TF combinations that regulate NB trans-differentiation; 2) aim 2 is to identify TFs and TF combinations that contribute to chemotherapy resistance of NB.

15. SUBJECT TERMS

Neuroblastoma, transcription factors, core regulatory circuitry, phenotypic switching, CRISPRa, single-cell RNA sequencing, CRISP-seq, perturb-seq

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1. INTRODUCTION:

Neuroblastoma (NB) is heterogenous, and it mainly includes adrenergic (ADRN) and mesenchymal (MES) tumor cell types. These NB subtypes are able to trans-differentiate or interconvert. The phenotypic and genotypic plasticity most likely contributes to the fact that many high-risk tumors initially respond to chemotherapy drugs but ultimately relapse. In this proposal, I will adapt recently developed single-cell CRISPRa sequencing technique to evaluate a novel concept of one or a group of TFs are essential in regulating NB cell phenotypic switching and chemotherapy drug resistance in NB

2. KEYWORDS:

Neuroblastoma, transcription factors, core regulatory circuitry, phenotypic switching, CRISPRa, single-cell RNA sequencing, CRISP-seq, perturb-seq

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 are two major goals of this project: 1, Identify transcription factors (TFs) and TF combinations that regulate neuroblastoma (NB) cells trans-differentiation; 2, Identify TFs and TF combinations that contribute to chemotherapy resistance of NB.

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.

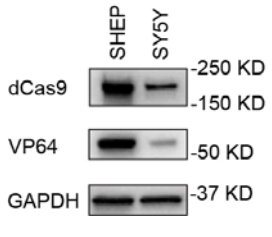

Figure 1. Verification NB-CRISPRa clones.

Aim 1. Identify transcription factors (TFs) and TF combinations that regulate neuroblastoma (NB) cells trans-differentiation.

Major Task 1. *Generating NB stable cell lines with the CRISPR system.*

By transducing dCas9-SunTag and scFv-VP64 lentiviral particles into ADRN-type SY5Y and MES-type SHEP cells, and selecting the cell with blasticidin and geneticin, I have established stable SY5Y-CRISPRa and SHEP-CRISPRa cell lines. The expression of dCas9 and VP64 was detected by western blot assay (Fig. 1).

Major Task 2. *Generating sgRNA libraries.*

I have designed two sgRNA libraries to target specific groups of TFs. The ADRN_sgRNAs_library is designed to target TFs are specifically expressed in ADRN-type of NB, while the MES_sgRNAs_library is aimed at TFs specifically expressed in MES-type of NB. These sgRNAs have been successfully cloned into dual-guide 3' direct-capture Perturb-seq vectors and verified through DNA sequencing. Additionally, I have generated lentiviral particles for both of these sgRNA libraries. As a result, Major Task 2 has been successfully accomplished.

Major Task 3. *Perform CRISPRa-seq to identify TFs that regulate NB cells phenotypic switching.*

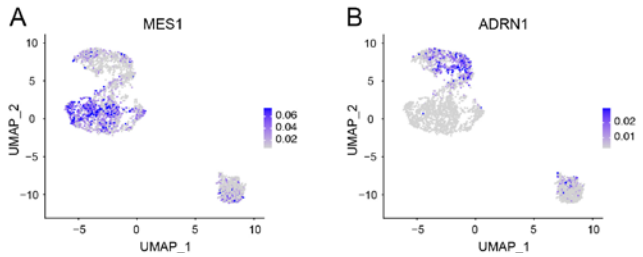

Figure 2. Single-cell RNA-seq analysis of SHEP-CRISPRa cells transduced with ADRN_sgRNAs_library. The UMAP plot in (A) shows MES signature scores, while in (B) it depicts ADRN signature scores within the SHEP-CRISPRa cell population.

To evaluate the efficacy of the CRISPRa-seq system, I performed a pilot single-cell RNA sequencing (scRNA-seq) experiment on SHEP-CRISPRa cells. These cells were transduced with the lentiviral ADRN_sgRNAs_library for a duration of 18 days. My expectation was that the TFs that specifically expressed in the ADRN-type of NB would be activated in MES-type SHEP cells through CRISPR activation. In the ADRN_sgRNAs_library, I also included several other TFs and important epigenetic modifiers that are implicated in cancer progression. For this scRNA-seq, a total of 3,000 high quality SHEP cells were captured. Through analysis of the ADRN and MES cell signatures as generated by Van Groningen et al. (DOI: 10.1038/ng.3899) within the scRNA-seq data, I identified three major cell populations. One population exhibited a high ADRN signature score, while another showed high MES signature score. These observations were indicated by UMAP plots (Fig. 2). Parental SHEP cells only showed homogenous cell population with high MES signature scores (data not shown). This result suggests the induction of an ADRN-like transcriptional profile in MES-type SHEP cells after activation of certain TFs.

Subsequently, we delved into the correlation between cells expressing the sgRNAs for each TF and their corresponding ADRN or MES signature scores. Of note, out of the 40 TFs that we are targeting, we identified that only 16 TFs-associated sgRNAs were expressed in a substantial number of cells (more than 50 cells) (Fig. 3A, B). This observation points to an uneven distribution of these sgRNAs within the library. Among these 16 TFs, we made a noteworthy discovery: cells expressing MYC sgRNAs exhibited a significantly lower MES signature score, but a higher ADRN signature score when compared to cells expressing non-targeting sgRNA controls (Fig. 3A, B). Furthermore, an elevation in MYC mRNA levels was evident in cells expressing MYC sgRNAs compared to those expressing control sgRNAs (Fig. 3C). Consistently, we also noticed that cells with elevated sgRNA reads consistently displayed heightened levels of the associated TFs (data not shown).

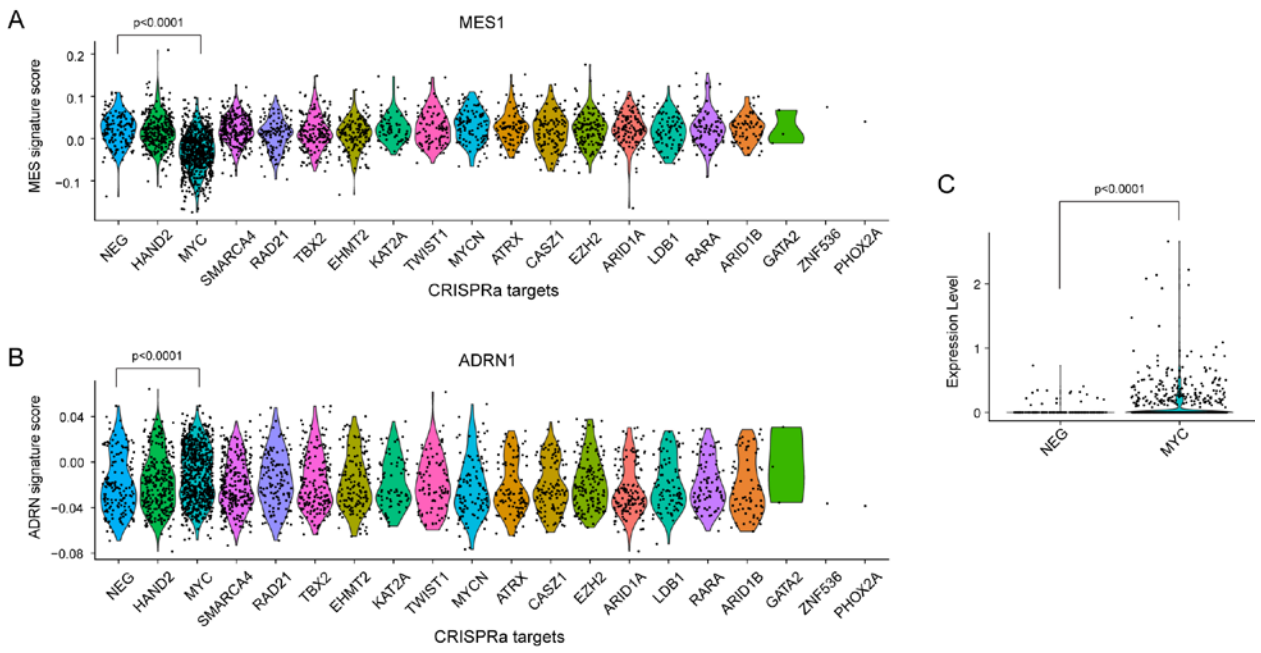

Figure 3. Single-cell RNA-seq analysis of SHEP-CRISPRa cells transduced with ADRN_sgRNAs_library. (A) MES signature scores of cells expressing sgRNAs targeting various TFs. (B) ADRN signature scores of cells expressing sgRNAs targeting various TFs. (C) MYC mRNA levels in cells expressing control sgRNAs or MYC sgRNAs.

Collectively, this pilot study demonstrates the capability of the CRISPRa-seq approach to activate target TF expression and to detect changes in associated transcriptional profiles. Notably, we have successfully identified MYC as a promising TF with the potential to induce transdifferentiation from MES-type to ADRN-type in SHEP cells. In our forthcoming study, we will take measures to ensure an even distribution of each sgRNA within the library. This can be achieved by manually generate each sgRNA construct and subsequently combining them into a library. Subsequently, we will conduct a more comprehensive CRISPRa-seq experiments using both SY5Y-CRISPRa and SHEP-CRISPRa cell lines as planed in the statement of work.

Specific Aim 2. Identify TFs and TF combinations that contribute to chemotherapy resistance of NB.

This task has not been started. I will start this task as soon as I finish the specific aim 1, major task 3.

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.

Currently, I have performed a pilot scRNA-seq experiments, and validated our CRISPRa-seq in SHEP-CRISPRa cell line. Next, I will work on major task 3 in a comprehensive way, which is to perform CRISPRa-seq to identify TFs that regulate NB cells phenotypic switching by infecting SHEP-CRISPRa and SY5Y-CRISPRa cell lines with the sgRNA libraries I generated. Subsequently, I will start major task 4, which is to perform CRISPRa-seq to identify TFs that protect NB cells from chemotherapy drugs.

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.

As described earlier in the pilot study, among the 40 targeted TFs, we found that only 16 TFs-associated sgRNAs were expressed in a considerable number of cells (Fig. 3A, B). This finding indicates an uneven distribution of these sgRNAs within the library. Moreover, although CRISPRa activated the expression of target TFs, the degree of activation was not substantial (Fig. 3C). Therefore, in addition to the CRISPRa-seq method, I intend to incorporate a barcoded open reading frame screening approach, as described in DOI: 10.1016/j.cell.2022.11.026. This approach involves overexpressing barcoded TF libraries in either SHEP cells or SY5Y cells, followed by scRNA-seq. Technically, this approach is more convenient and simpler than the CRIPSRa-seq method. By integrating this approach, I anticipate a higher successful rate for this project.

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.

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: *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.

Nothing to report.

- **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.

Nothing to report.

- **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.*

Nothing to report.

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.)*

Name:	Zhihui Liu
Project Role:	Principal Investigator
Nearest Person month worked:	12 calendar months
Contribution to Project:	Dr. Liu is responsible for the execution of the entire SOW for this project.

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.

Nothing to report.

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: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: *N/A*