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14. ABSTRACT

Despite current aggressive regimens, the majority of patients with MYCN amplification die due to drug-resistant disease, and further intensification of chemotherapy will not significantly improve this outcome. We propose an entirely novel strategy to oppose MYCN oncogenic function in NB: by blocking the metabolic reprogramming driven by MYCN. Based on our data and the recent literature, our guiding hypotheses are that: a) lipid metabolism is required for NB tumorigenesis, and b) targeting MYCN-driven lipogenesis will effectively block NB tumor growth. We have demonstrated that lipid metabolism is a selective metabolic dependency of MYCN-driven tumors. MYCN drives both fatty acid (FA) synthesis and FA uptake to maintain NB cell survival. Targeting FA uptake effectively blocks NB in vivo tumor growth.

15. SUBJECT TERMS
Neuroblastoma, MYCN, fatty acid metabolism, targeted therapies.

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1. INTRODUCTION:

Our long-term goal is to elucidate the MYCN-dependent pathways that will serve as targets for NB therapy. Toward this goal, our overall objective in this application is to determine **how MYCN rewires lipid metabolism to support tumor growth**. We hypothesize that: a) lipid metabolism is required for NB tumorigenesis, and b) targeting MYCN-driven lipogenesis will effectively block NB tumor growth. In this proposal we will: **1)** Determine how MYCN reprograms lipid metabolism in NB, and **2)** Elucidate the anti-tumor activity of targeting MYCN-driven lipogenesis. These studies will reveal insights into critical molecular and metabolic alterations, which will provide novel, and more sensitive targets that could be deployed with currently available therapies to treat this highly aggressive disease.

2. KEYWORDS:

Neuroblastoma (NB)
V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN)
Lipid metabolism
Fatty acids (FA)
Tumorigenesis
Targeted therapies
Fatty Acid Transport Protein 2 (FATP2)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine how MYCN reprograms lipid metabolism in neuroblastoma.
1.1 Metabolic *in vitro* characterization upon changes in MYCN expression.
1.2 *In vitro* effects of genetic interference and pharmacological inhibition of lipogenesis.
1.3 Elucidate how MYCN alters *in vivo* lipid metabolism and tumor growth.

Specific Aim 2: Elucidate the anti-tumor activity of targeting MYCN-driven lipogenesis.
2.1 *In vivo* anti-tumor activity of targeting lipogenesis via single agent FASN and FATP2 inhibitors.
2.2. *In vivo* anti-tumor activity of targeting fatty acid synthesis and uptake.
2.3 Determine how inhibition of lipogenesis alters *in vivo* chemo-sensitivity.

What was accomplished under these goals?

Specific Aim 1: Determine how MYCN reprograms lipid metabolism in neuroblastoma.

1. Metabolic *in vitro* characterization upon changes in MYCN expression

1.1 We have evaluated cell proliferation (MTT and Brdu assays) in MYCN-amplified cells upon genetic depletion of MYCN and we have confirmed that MYCN offers cell growth advantages to neuroblastoma (NB) cells. As predicted, efficient knockdown of MYCN significantly reduces NB cell growth (data not shown).

1.2 We have validated cell lipid content by BODIPY$^{493/503}$ and oil red O staining in NB cells upon genetic depletion of MYCN (preliminary data of our proposal). We then extended our findings to a panel of non-MYCN amplified and MYCN-amplified cell lines, and SK-N-AS MYCN-ER cells. SK-N-AS MYCN-ER cells lack amplification of MYCN and stably expresses inducible wild-type MYCN-ERTM (estrogen receptor tamoxifen mutant). When stimulated with 4-hydroxytamoxifen (4OHT), MYCN-ER translocates into the nucleus and upregulates MYCN targets) **(Figure 1)**. Our data suggest that **MYCN promotes lipid accumulation in NB cells**.

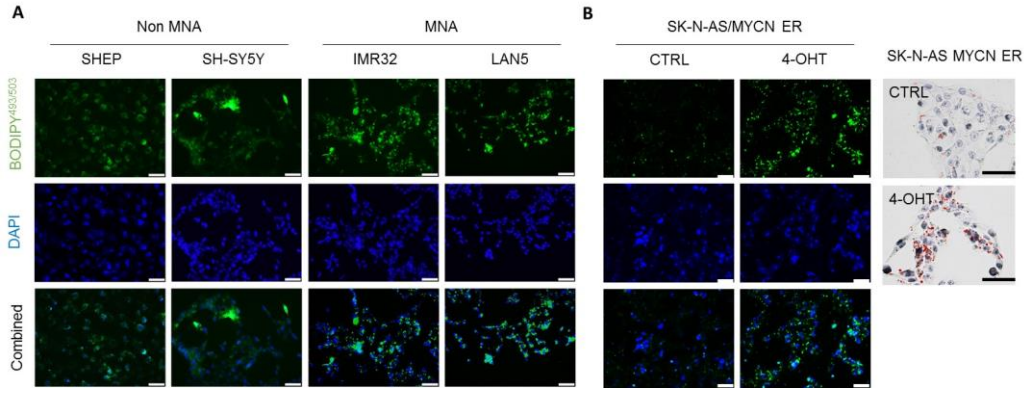

Figure 1: A. BODIPY493/503 with and without DAPI staining in non MYCN-amplified (non MNA, SHEP and SH-SY5Y) and –amplified (MNA, IMR32 and LAN5) NB cells. **B.** BODIPY493/503 with and without DAPI staining and oil red O staining in SK-N-AS MYCN-ER cells with and without MYCN activation (4-OHT).

1.3 We have evaluated fatty acid (FA) transfer and synthesis by 13C-isotope tracing in MYCN-driven cells upon changes in MYCN expression. Stable isotope D2O and palmitate[U-13C] were employed to trace FA synthesis and uptake in two independent MYCN-driven models. MYCN drives both FA synthesis and FA uptake in MYCN3 (MYCN Tet-On) and SK-N-AS MYCN-ER cells (-/+ MYCN activation). Our data suggest that **MYCN drives both FA synthesis and uptake in NB (Figure 2A and 2B)**. Importantly, **FA uptake is necessary for MYCN-amplified cell survival**, as deprivation of exogenous FAs blocks cell proliferation ($p<0.01$) **(Figure 3)** and induces apoptotic cell death (data not shown).

We are currently assessing FA transfer and synthesis (by BODIPY-C12 staining) in NB cells both in normoxic and hypoxic conditions upon changes in MYCN expression.

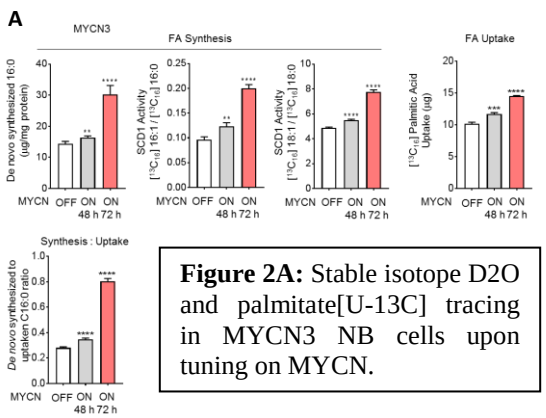

Figure 2A: Stable isotope D2O and palmitate[U-13C] tracing in MYCN3 NB cells upon tuning on MYCN.

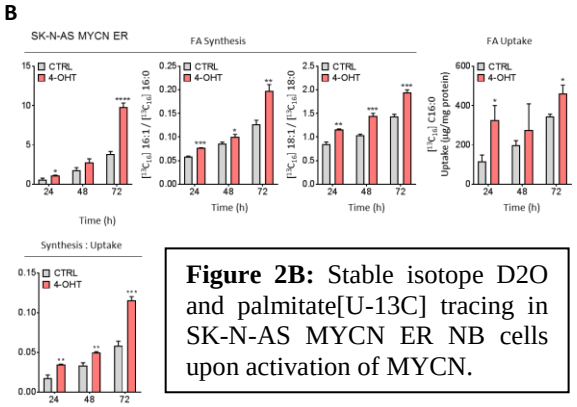

Figure 2B: Stable isotope D2O and palmitate[U-13C] tracing in SK-N-AS MYCN ER NB cells upon activation of MYCN.

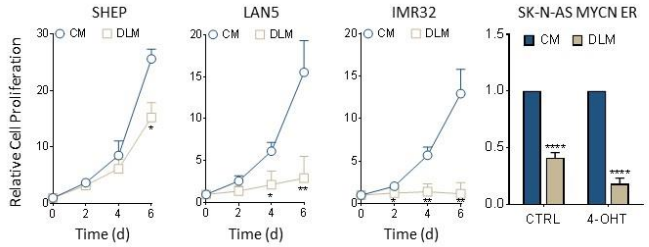

Figure 3: Cell proliferation (CCK-8 assay) in non MYCN-amplified (SHEP) and MYCN-amplified cells (LAN5 and IMR32) upon deprivation of exogenous lipids (delipidized media – DLM). Effect of DLM was also assessed in SK-N-AS MYCN ER cells upon activation of MYCN (4-OHT).

2. *In vitro* effects of genetic interference and pharmacological inhibition of lipogenesis

2.1 and 2.2. We have evaluated how pharmacological targeting of lipogenesis alters cell growth and lipid metabolism. The small molecule SCD1 inhibitor A939572 effectively inhibits FA synthesis in MYCN-amplified NB cells (**Figure 4A**) and blocks cell growth in a panel of NB lines (**Figure 4B**). Interestingly, inhibition of FA synthesis by A939572 induces a compensatory uptake of FA, which is essential for NB cell growth **(Figure 4C)**. This suggests that **FA uptake may represent a key mechanism for NB cells to maintain their high proliferative state**.

We are currently generating NB cells with conditional shRNA against the lipogenic enzymes FASN and SCD1. We predict that both pharmacological inhibition and genetic depletion of these lipogenic enzymes will oppose the cell growth induced by MYCN.

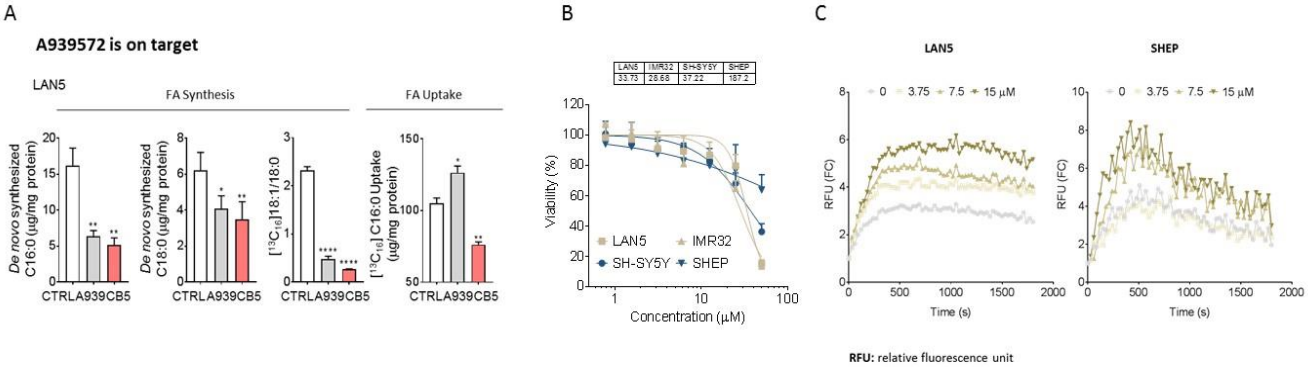

Figure 1: A. Stable isotope D2O and palmitate[U-13C] tracing in LAN5 cells upon inhibition of lipogenesis via the small molecule SCD1 inhibitor A939572 (A939) and the inhibitor of FA uptake CB5. **B.** Cell viability assay (MTT) in a panel of NB cell lines upon treatment with A939572 (IC_{50} are shown in the table). **C.** Treatment with increasing doses of A939572 induces a compensatory FA uptake in NB cells. Real-time FA uptake was analyzed using the QBT Fatty Acid Uptake Assay Kit (Molecular Devices). After 48h of serum starvation, cells were treated with 0–15 uM A939572 for 15 min before the assay reagent was added. Plate was read at λex = 485 nm and λem = 515 nm every 30s for 30min. Data are expressed as relative fluorescence unit fold change to time "0".

3. Elucidate how MYCN alters *in vivo* lipid metabolism and tumor growth

We have not yet elucidate how MYCN alters *in vivo* lipid metabolism and tumor growth. This objective has not yet started due to the suspension of animal work caused by Covid-19.

Specific Aim 2: Elucidate the anti-tumor activity of targeting MYCN-driven lipogenesis.

4. *In vivo* anti-tumor activity of targeting lipogenesis via single agent FASN and FATP2 inhibitors

We have compared the *in vivo* anti-tumor activity of the FASN inhibitor (orlistat) and the FATP2 inhibitor (CB5) in MYCN-amplified LAN5-derived xenografts, and we have demonstrated that both these agents are able to significantly reduce tumor growth in this model of NB (**Figure 5 A**). Because of our preliminary data showing compensatory uptake of FAs upon inhibition of lipogenesis, we then validate the anti-tumor activity of CB5 as single agent in additional MYCN-driven NB models. **CB5 was able to significantly inhibit tumor growth** in a TH-MYCN allograft model (xenografts derived from TH-MYCN+/+ NB tumors) (**Figure 5 B**) and prolong animal survival in a MYCN-amplified patient-derived model (PDX, collected here at TCH) (**Figure 5 C**).

We are currently evaluating the anti-cancer activity of these agents in non MYCN-amplified NB models and determining their effects on *in vivo* lipid metabolism.

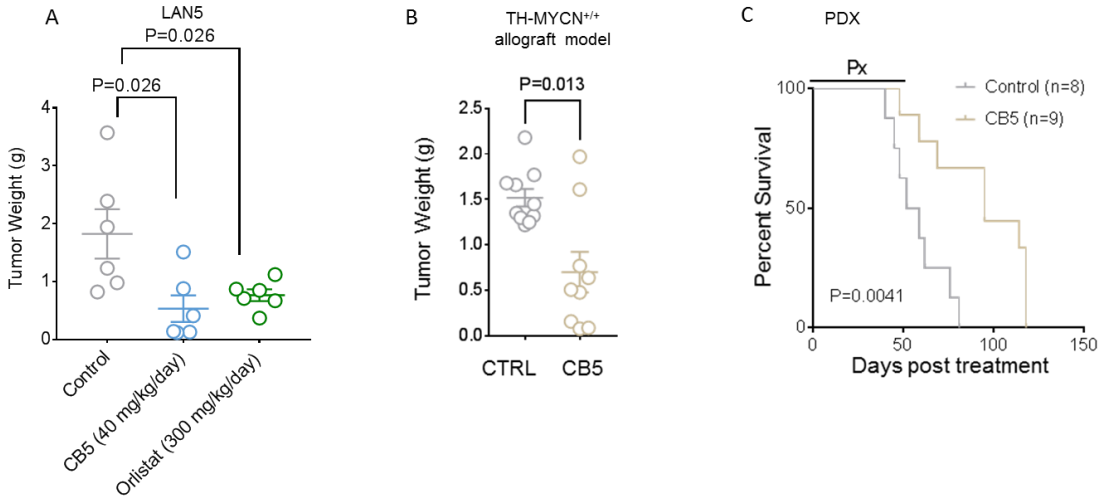

Figure 5: A. Anti-tumor activity of orlistat and CB5 in LAN5-derived NB xenografts. After tumor establishment, treatment was continued for three weeks at the indicated doses. TW (g) are shown. **B.** Anti-tumor activity of CB5 in THMYCN-derived allografts (derived from TH-MYCN+/+ NB tumors). After tumor establishment, treatment was continued for three weeks. TW (g) are shown. **C.** Anti-tumor activity of CB5 in a MYCN-amplified patient derived xenograft (PDX). Treatment was continued for three weeks (from week 3 to week 7). Kaplan-Meier survival analysis (percent survival) is represented.

5. *In vivo* anti-tumor activity of targeting fatty acid synthesis and uptake

We have not started testing the anti-tumor activity of combined FASN and FATP2 inhibition in MYCN cell-derived xenografts and PDXs. This goal has not yet started due to the suspension of animal work caused by Covid-19.

6. Determine how inhibition of lipogenesis alters *in vivo* chemo-sensitivity

We have optimized *in vitro* drug concentrations for our combination therapy (VP16 + CB5) and we have demonstrated that this combination synergistically inhibits NB cell growth selectively in MYCN-amplified cells, suggesting that **this metabolic vulnerability is MYCN-dependent** (**Figure 6**).

We are currently testing the effect of this combination therapy in MYCN-amplified xenografts and patient-derived xenografts.

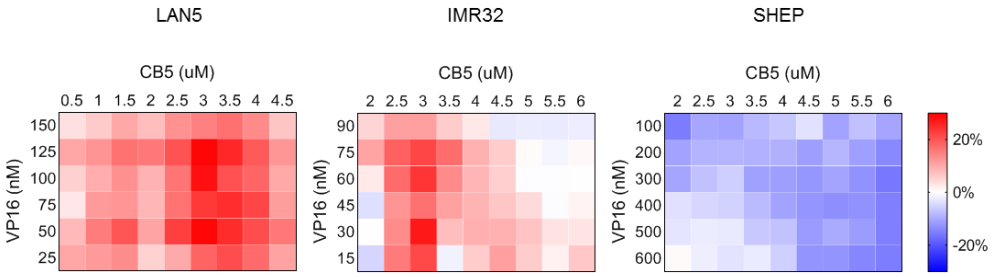

Figure 6: Cell viability (MTT assay) in MYCN-amplified (LAN5 and IMR32) and non MYCN-amplified (SHEP) cells upon different concentrations of CB5 and VP16. If difference between observed inhibition rates and predictive (Bliss) inhibition rates are >0.1 (10%) = synergistic effect; <0.1 (10%) = antagonistic effect; and between -0.1 and 0.1 = additive effect.

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

Ling Tao Ph.D., post-doctoral associate (100% effort supported by this grant), will continue to learn new lab skills necessary for this project, including stable isotope tracing, FA profiling, and additional *in vivo* models. These skills will expand her expertise both in the field of molecular biology and cancer metabolism, and will enable her to identify distinct metabolic phenotypes and novel MYCN targets to pursue in future research efforts. Throughout the project, she will also develop critical skills that will help foster her academic career. To improve her writing and data analysis skills, and expand her knowledge on translational research she will attend the BCM courses on Scientific Writing, Explorative Data Analysis, and Translational Cancer Biology. Recently, she have trained one SMART program undergraduate student and one visiting postdoctoral fellow. She has also taught the BCM Molecular Refresher Course on different topics (i.e. cell cycle and chromosome stability, and transcription regulation and RNA sequencing). She will continue training junior members of the lab and giving lectures to students within the BCM community. She had leadership experience during the organization of the BCM Annual Career Symposium for postdoctoral fellows, and she will continue expanding her leadership skills by participating in the

organization of the Texas Medical Center (TMC) Annual Postdoctoral Symposium. She will also present her work at the Texas Children's Hospital (TCH) Neuroblastoma Work in Progress meetings every 6 months, the TCH Research Symposium annually, and national/international conferences, including keystone symposiums, and AACR and ANR (Advances in Neuroblastoma Research) meetings.

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?

As part of aim1, we are currently generating NB cells with conditional shRNA against major lipogenic enzymes (such as FASN, SCD1, and CB5). We will test the contribution of these enzymes to NB cell phenotype and lipid metabolism. We will also elucidate how genetic depletion of MYCN alters *in vivo* lipid metabolism and tumor growth.
As part of aim2, we are currently evaluating the anti-tumor activity of orlistat and CB5 in non MYCN-amplified models and determining their consequences on *in vivo* lipid metabolism. We will also determine how FATP2 inhibition alone and in combination with *de novo* lipid synthesis inhibitors alter NB *in vivo* sensitivity to conventional chemotherapy (VP16).

4. IMPACT:

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

MYCN amplification occurs in half of high-risk NB patients and strongly correlates with disease progression and treatment failure. Hence, there is an unmet need to identify novel MYCN-dependent pathways and develop effective therapies for high-risk patients. The MYC oncogene is well documented as a master regulator of cell metabolism to support tumor growth. However, how MYCN reprograms NB tumor metabolism and its impact on tumor growth remain elusive. The overall goal of our study was to identify novel MYCN-driven metabolic alterations that contribute to NB oncogenesis. **By selectively targeting specific metabolic dependencies, we will be able to identify innovative and effective therapeutic approaches for high-risk disease**.
Our data reveal a novel metabolic dependency of MYCN-amplified tumors: MYCN activates lipid metabolism and specifically drives fatty acids uptake to support tumor growth. Pharmacological inhibition of fatty acids uptake effectively blocks tumor growth and sensitizes NB cells to conventional therapy.

What was the impact on other disciplines?

Our rationale for the proposed research is that it will reveal novel pathways and modes of regulation that will provide us with new and more sensitive therapeutic targets for MYCN amplified NBs. This will also provide a novel and important approach to intervention for the many human cancers that utilize MYC for oncogenesis. More broadly, we expect that the proposed research will provide new insight into the regulation of energy metabolism in cancer progression, with implications for metabolic syndromes and other human diseases.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

This approach is exciting because it elucidates novel MYCN-dependent pathways that will serve as targets for NB treatment. Finding novel effective targeted therapies that could be safely included in current regiments for relapse disease has enormous clinical implications.

5. CHANGES/PROBLEMS:

Nothing to Report.

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

The proposed animal work is currently delayed due to the suspension of animal work and reduced lab capacity caused by Covid-19. We are currently at 50% lab capacity and animal work has now resumed. Animal work plan may change if new restrictions will take place in our institution.

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

Not applicable.

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**

  Journal publications.

  Nothing to Report.

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

Nothing to Report.

Other publications, conference papers and presentations.

- Virtual 2021 ANR. Advances in Neuroblastoma Research (ANR) webinar January 25-26- 27, 2021; https://www.anr2021.org. Oral communication.

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

  Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Eveline Barbieri, MD PhD, PI – no changes

Ling Tao, PhD, Postdoctoral Associate – no changes

Mirthala Moreno Smith, PhD, Research Associate – no changes

Nagireddy Putluri, PhD, Co-Investigator – no changes

Sanjeev A. Vasudevan, MD, Co-Investigator – no changes

Cristian Coarfa, PhD, Co-Investigator – no changes

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?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable.

QUAD CHARTS:

Not applicable.

9. APPENDICES:

Not applicable.