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TITLE

Precision Targeting of TFAP-2 for Melanoma Heterogeneity Using CRISPR Containing Lipid Nanoparticles

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SUMMARY

This project envisions addressing a significant gap in the therapeutic landscape for melanoma due to the inefficacy of conventional chemotherapy, radiotherapy, and small molecule inhibitors. To address this challenge, this research proposes a novel approach utilizing CRISPR gene editing technology delivered via lipid nanoparticles (LNPs) or exosomes to target the TFAP2 gene in melanoma cells. We hypothesized that the TFAP2 targeting in melanoma should alter the tumor microenvironment by reducing innervations and extracellular matrix sensing of melanoma cells (Figure 6). Preliminary experiments have focused on the TFAP2 isoform TFAP2A, which has been found to be overexpressed in melanoma and is associated with poor overall and disease-free survival in patients. Knockout experiments using the SkMel28 melanoma cell line have demonstrated that TFAP2A knockdown effectively downregulates pathways involved in neuronal differentiation and extracellular matrix sensing, both critical factors in melanoma tumorigenesis. This research paper outlines the design of guide RNA (gRNA) sequences for targeting TFAP2 in melanoma cells and emphasizes the use of LNPs as a safe and precise delivery mechanism for in vivo gene editing.

INTRODUCTION

Cancer is a complex and multifaceted disease that has plagued humanity for centuries. It is characterized by the uncontrolled growth of abnormal cells that can invade and destroy healthy tissues (1). Cancer is a major global health concern, accounting for an estimated 10 million deaths worldwide in 2020. In the United States, cancer statistics are alarming. In 2020, 1,603,844 new cancer cases were reported and 602,347 people died of cancer. For every 100,000 people, 403 new cancer cases were reported and 144 people died of cancer (1). The

mortality rate for all types of cancer was 189.5/100,000 for males, and 135.7/100,000 for females (2,3). Among different types of cancers, skin cancer is the most common in the US. Melanoma, also known as malignant melanoma, is a type of skin cancer that develops from the pigment-producing cells known as melanocytes. It is the most serious type of skin cancer because it often spreads to other parts of the body if not caught early. In the United States, there are an estimated 187,000 new cases expected in 2023 (4), and its impact spans beyond the skin, affecting various body areas like the eyes, scalp, and even nails. Unlike some cancers, melanoma doesn't discriminate based on age, race, or gender - however, in the 30-39 age group, melanoma ranks as the fifth most diagnosed cancer. The incidence among individuals under 30 has surged by 50% in women since 1980, and approximately 400 children are diagnosed annually. While the rates have doubled between 1982 and 2011, they continue to rise, affecting around 1.3 million Americans today (4). The risk varies among ethnicities, with Caucasians facing a higher lifetime risk compared to Hispanics and African Americans. Additionally, melanoma can manifest in unexpected areas, making diagnosis challenging, especially among people of color where it's often discovered in advanced stages, complicating treatment (4). Lesser-known variations like ocular and mucosal melanomas contribute to its complexity, with ocular melanoma being the most prevalent eye tumor in adults, and mucosal melanoma accounting for about 1% of cases, which can uniquely challenge diagnosis and management (5).

Research question that we ask is that if we can we create a more personalized and effective treatment to tumor metastasis and provide an accessible, affordable, and sustainable melanoma treatment via addressing tumor heterogeneity by manipulating the features of tumors themselves?

We hypothesize that the TFAP2 targeting in melanoma should alter the tumor microenvironment by reducing innervations and extracellular matrix sensing of melanoma cells (Figure 6).

The approach that we have taken is that the non-viral delivery methods - lipid nanoparticles (LNPs) should be utilized for targeting melanoma TME and TFAP2. The advantages such an approach would offer are long term treatment, safety, cost, potential for uptake and precision.

The research has offered valuable insight that TFAP2 gene is differentially over expressed in melanoma compared to normal. Targeting TFAP2 gene can lead to poor innervation in melanoma. The CRISPR construct along with the gRNA target with LNP's surface can be transported modified with ligands or antibodies that recognize and bind to specific markers or receptors uniquely expressed on cancer cells. This targeted binding increases the likelihood of

preferential uptake by cancer cells while reducing uptake by healthy cells in the TME. TFAP2 targeting in melanoma is an effective and more long-lasting solution to treating melanoma.

RESULTS

Utilizing GEPIA server (https://gepia.cancer-pku.cn/), I found that the TFAP2A is the most critical one with huge differential expression in melanoma (Figure 1) among different isoforms (Figures 1 and 2). Along with that, we have observed that the TFAP2A expression is directly altering the overall and disease-free survival (Figure 1B and Figure 1C). In melanoma (TCGA-SKCM) the gene is highly overexpressed compared to normal (Figure 2A). TFAP2A gene expression is correlated to overall and disease-free survival in melanoma (Figures 2B and 2C). TFAP2A, which is also a lineage specific transcription factor for melanocytes development, has orchestrated cellular and molecular heterogeneity in the tumor microenvironment. We observed TFAP2 knockout using CRISPR has downregulated a cluster of pathways related to neuronal differentiation, neurogenesis, and neuro development (Figure 3B). It also altered genes sensing extracellular matrix. All the TFAP2 subtypes are not significantly over-expressed in melanoma compared to normal.

Knockout experiments using the SkMel28 melanoma cell line have demonstrated that TFAP2A knockdown effectively downregulates pathways involved in neuronal differentiation and extracellular matrix sensing, both critical factors in melanoma tumorigenesis. The TME is altered to become less heterogeneous and non-metastatic. The data for some selective genes responsible for melanoma growth, signaling, transcriptional network, and microenvironment complexity is analyzed. It is observed after TFAP2 knockout that the log2FC of the targeted gene list has drastically decreased. It suggests that TFAP2 can be a good target for the Cas9 delivery system. Different TFAP2 isoforms are observed, and it is seen that TFAP2A is the most significantly overexpressed and regulating survival, moving towards design of in-vivo knockout via Cas9/LNP delivery. Knockout experiments using the SkMel28 melanoma cell line have demonstrated that TFAP2A knockdown effectively downregulates pathways involved in neuronal differentiation and extracellular matrix sensing, both critical factors in melanoma tumorigenesis, as seen with the bioluminescence imaging of the thoracic and abdominal metastasis and subsequent knockout with the eliminated metastases. The TME is altered to become less heterogeneous and non-metastatic.

DISCUSSION

Because melanoma is so differential, there are limited treatment/therapeutic options and a greater chance for therapy resistance. These differentials can be brought down to the tumor microenvironment (TME) (6,7). Among different factors that make targeting the tumors very hard, one of the key components is TME. The TME is the intricate and dynamic environment that surrounds a tumor, particularly a solid tumor (8,9,10). It comprises a complex network of cells, including non-tumor cells, extracellular matrix (ECM) components, and soluble factors, that play a crucial role in tumor progression, metastasis, and treatment resistance. TME has immune cells, nerve fibers, adipocytes, cancer-associated fibroblasts, endothelial cells, pericytes, and various microorganisms like fungi, bacteria, etc. (8,11,12). With the TME, a fundamental aspect of this is its heterogeneity - the presence of The TME plays a crucial role in tumor growth, progression, and response to therapy. Heterogeneity in the TME is the presence of distinct molecular and cellular characteristics among different tumors and even within the same tumor, which can impact the tumor's behavior and response to treatment. The origin of heterogeneity also varying like cellular heterogeneity (different tumor or immune cells composition), molecular heterogeneity (molecular difference among a particular cell type, let's say cancer cells, in different regions of the tumor), genomic heterogeneity (due to random mutations all the cancer cells harbor different mutations and genomic alterations and that vary from cell to cell), or bio-physical heterogeneity (assume a mass of cells are growing uncontrollably and the physical parameters like force, tension, flow of oxygen, etc. are different in core, periphery and intermediate zone of the tumor) (13,14,15,16). One of the crucial components of the TME is peripheral nerve fibers. The innervation of TME is associated with aggressive tumor phenotype and poor prognosis (17,18). These sympathetic and parasympathetic neural effects are orchestrated by β-adrenergic or muscarinic receptors and can be explained by changes in cancer cell behavior like migration, ECM sensing, angiogenesis, tumor-associated macrophages, and adaptive antitumor immunity. These nerve fibers also alter electrochemical properties of the microenvironment (19,20). By stimulating angiogenesis through vascular endothelial growth factor signaling, intratumoral adrenergic neurons release noradrenaline, which accelerates the growth of tumors. Intratumoral parasympathetic neurons could play a dual function in the advancement of cancer by triggering Wnt-β-catenin signals that proliferate cancer stem cells. Therefore, targeting pathways related to innervation might be a broad-spectrum approach against solid tumors (20,21). On the other hand, melanoma displays molecular diversity due to various genetic mutations, impacting tumor behavior and treatment responses. Tumor heterogeneity in melanoma arises from mutations in genes like BRAF, NRAS, and PTEN, affecting cell growth and survival pathways (22). All the

heterogeneity interplays using different transcriptional networks via a plethora of transcription factors.

Cis-responsive elements (CREs) play a crucial role in gene regulation, acting as binding sites for transcription factors (TFs) that control the expression of nearby genes. These elements are involved in diverse cellular processes, including development, differentiation, and response to environmental stimuli. By interacting with specific TFs, CREs facilitate the precise spatial and temporal regulation of gene expression, allowing cells to adapt to varying conditions and signals(23,24,25). In this way, CREs contribute to the intricate orchestration of biological processes, ensuring the proper functioning and homeostasis of living organisms. Transdifferentiation, the process by which one cell type is converted directly into another without going through a pluripotent state, heavily relies on the activation or repression of specific genes. TFs play a crucial role in trans differentiation by orchestrating the complex gene regulatory networks required for cellular reprogramming. They can induce the expression of lineage-specific genes while repressing the expression of genes characteristic of the original cell type (26,27). This orchestrated gene expression change is essential for the successful conversion of one cell type into another, as it involves a drastic shift in cellular identity and function. Transcription Factor AP-2 (TFAP2) plays a role in melanoma by regulating genes involved in cell differentiation and development. TFAP2's influence on cellular processes underscores its significance in melanoma progression and response to therapies (28). Understanding this molecular diversity and TFAP2's involvement offers insights into designing targeted treatments for different melanoma subtypes, crucial for improving patient outcomes. TFAP2 is a lineage specific transcription factor found on chromosome 6. A study by Geeta *et al* on ECM1 regulation by TFAP2 gives a lot of insight into the role of TFAP2 within melanoma (29). In melanoma, TFAP2 appears to transcriptionally sense the extracellular matrix (ECM) by regulating the expression of Extracellular Matrix 1 -ECM1 (5,28). The study reveals that ECM1 is overexpressed in melanoma cell lines compared to primary melanocytes (28). Its expression correlates with TFAP2C levels, and the knockdown of TFAP2C leads to a reduction in ECM1 expression, while TFAP2C upregulation results in ECM1 upregulation. With 5' RACE and luciferase reporter assays, the minimal promoter region of human ECM1 is identified. The P2 regulatory region in the ECM1 promoter, confirmed by gel-shift assays demonstrating TFAP2C binding to this site. ECM1 knockdown affects melanoma cell attachment, consistent with the association of ECM1 overexpression with poor prognosis (28,29,30). We can then see the role of TFAP2C in melanoma, highlighting its involvement in ECM1 regulation and emphasizing the

intricate transcriptional control of ECM-related processes in melanoma progression which is why this is an important target within the context of melanoma (29). I am using non-viral delivery methods, specifically lipid nanoparticles (LNPs), for targeting melanoma and TFAP2 due to their potential advantages in precision and safety. LNPs are specialized carriers designed to encapsulate mRNA encoding Cas9 and guide RNA, delivering these gene-editing components into cells. To specifically target melanoma and minimize off-target effects within the TME, the LNP's surface can be modified with ligands or antibodies that recognize and bind to specific markers or receptors uniquely expressed on cancer cells (31). This targeted binding increases the likelihood of preferential uptake by cancer cells while reducing uptake by healthy cells in the TME, potentially enhancing the precision of gene editing for melanoma or TFAP2-related therapies.

Personalized medicine in cancer therapy combines CRISPR gene editing with genomic analysis for tailored treatment regimens and maximum therapeutic efficacy is the future. Recent approvals like Casgevy for sickle cell disease highlight CRISPR's potential; future refinement promises widespread accessibility and effectiveness in therapy. Future cancer therapy integrates CRISPR-dCas9 ATFs with repurposed drugs, enhanced by engineered Cas9 variants and precise guide RNA selection. There are some limitations; In certain cancer cells, mutations in the sgRNA homology sequence or protospacer adjacent motif (PAM) could render the therapy ineffective, though is unlikely if conditions are ideal.

MATERIALS AND METHODS

Different isoforms of TFAP2 genes were curated from literature and their expression was checked in various cancers including melanoma The GEPIA server general and survival data was used to look at differential expression and survival of different TFAP2 isoforms. To understand the role of TFAP2A, query dataset with ID GSE190610 from NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) was selected. Different roles of TFAP2 in this dataset were studied using bulk genomics techniques - CUT&RUN, ATACseq and RNAseq. The wild type and TFAP2 knockout SkMel28 cell line data are compared. SkMel28 cell line is a widely studied melanoma cell line. From the dataset, a differentially expressed gene list between TFAP2 knockout SkMel28 vs wild type SkMel28 was fetched. Fold change with -3 or more were considered as down-regulated genes and those were selected for further analysis into the delivery system. As a first step of designing CRISPR guide RNA, the CRISPick server (https://portals.broadinstitute.org/gppx/crispick/public) from Broad Institute was used. For

knocking out the TFAP2A gene, two sgRNA with NGG PAM sequence for SpyoCas9 was selected. From the given guide RNA sequences, two were be picked based on their on-target and off-target score. One on the plus strand and the other one on the minus strand is selected. The two sgRNAs that are analyzed for are GGCCCACACGCGCTCAGCTC (sense strand, on-target efficacy score 0.5446) and GGAGTAAGGATCTTGCGACT (antisense strand, on-target efficacy score 0.4073). As a second step of preparation and delivery of LNP Constructs, the sgRNAs were delivered in the form of RNP complex using LNP. The Cas9 prepared using thermo stable Cas9 from Geobacillus stearothermophilus derived site-directed mutagenesis mediated evolved iGeoCas9, an alternate Cas9 to increase specificity. For in vivo and therapeutic usage, GMP grade LNP can be produced. LNP mediated co-delivery of iGeoCas9 RNPs can be personalized as per the metastatic nature of the cancer. The delivery method using the LNP method will be optimized based on collection.

REFERENCES:

1. “Cancer Data and Statistics." *Centers for Disease Control and Prevention.* www.cdc.gov/cancer/dcpc/data/index.htm. Accessed 13 Mar. 2024.
2. Brown, Joel S., et al. “Updating the Definition of Cancer.” *Molecular Cancer Research*, vol. 21, no. 11, Nov. 2023, pp. 1142–47. https://doi.org/10.1158/1541-7786.MCR-23-0411.
3. Sung, Hyuna, et al. “Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.” *CA: A Cancer Journal for Clinicians*, vol. 71, no. 3, May 2021, pp. 209–49. https://doi.org/10.3322/caac.21660.
4. “Facts & Stats - Melanoma Research Foundation.” *Melanoma Research Foundation.* www.melanoma.org/melanoma-education/understand-melanoma/facts-stats/. Accessed 13 Mar. 2024.
5. Patel, Meet, et al. “Resistance to Molecularly Targeted Therapies in Melanoma.” *Cancers*, vol. 13, no. 5, Mar. 2021, pp. 1115. https://doi.org/10.3390/cancers13051115.
6. Falcone, Italia, et al. “Tumor Microenvironment: Implications in Melanoma Resistance to Targeted Therapy and Immunotherapy.” *Cancers*, vol. 12, no. 10, Oct. 2020, p. 2870. https://doi.org/10.3390/cancers12102870.
7. Giraldo, Nicolas A., et al. “The Clinical Role of the TME in Solid Cancer.” *British Journal of Cancer*, vol. 120, no. 1, Jan. 2019, pp. 45–53. https://doi.org/10.1038/s41416-018-0327-z.

8. Baghban, Roghayyeh, et al. “Tumor Microenvironment Complexity and Therapeutic Implications at a Glance.” *Cell Communication and Signaling*, vol. 18, no. 1, Dec. 2020, pp. 59. https://doi.org/10.1186/s12964-020-0530-4.
9. Valkenburg, Kenneth C., et al. “Targeting the Tumour Stroma to Improve Cancer Therapy.” *Nature Reviews Clinical Oncology*, vol. 15, no. 6, June 2018, pp. 366–81. https://doi.org/10.1038/s41571-018-0007-1.
10. De Visser, Karin E., et al. “The Evolving Tumor Microenvironment: From Cancer Initiation to Metastatic Outgrowth.” *Cancer Cell*, vol. 41, no. 3, Mar. 2023, pp. 374–403. https://doi.org/10.1016/j.ccell.2023.02.016.
11. Alam, Aftab, et al. “Fungal Mycobiome Drives IL-33 Secretion and Type 2 Immunity in Pancreatic Cancer.” *Cancer Cell*, vol. 40, no. 2, Feb. 2022, pp. 153-167. https://doi.org/10.1016/j.ccell.2022.01.003.
12. Runa, F., et al. “Tumor Microenvironment Heterogeneity: Challenges and Opportunities.” *Current Molecular Biology Reports*, vol. 3, no. 4, Dec. 2017, pp. 218–29. https://doi.org/10.1007/s40610-017-0073-7.
13. Abramson, Vandana G., et al. “Molecular Heterogeneity of Triple-Negative Breast Cancer.” *Current Breast Cancer Reports*, vol. 6, no. 3, Sept. 2014, pp. 154–58. https://doi.org/10.1007/s12609-014-0152-1.
14. Ge, Rongbin, et al. “Tumor Microenvironment Heterogeneity an Important Mediator of Prostate Cancer Progression and Therapeutic Resistance.” *Npj Precision Oncology*, vol. 6, no. 1, May 2022, pp. 31. https://doi.org/10.1038/s41698-022-00272-w.
15. Sobral, Daniel, et al. “Genetic and Microenvironmental Intra-Tumor Heterogeneity Impacts Colorectal Cancer Evolution and Metastatic Development.” *Communications Biology*, vol. 5, no. 1, Sept. 2022, pp. 937. https://doi.org/10.1038/s42003-022-03884-x.
16. Gysler, Stefan M., et al. “Tumor Innervation: Peripheral Nerves Take Control of the Tumor Microenvironment.” *Journal of Clinical Investigation*, vol. 131, no. 11, June 2021, pp. e147276. https://doi.org/10.1172/JCI147276.
17. Li, Xinyu, et al. “Targeting Tumor Innervation: Premises, Promises, and Challenges.” *Cell Death Discovery*, vol. 8, no. 1, Mar. 2022, pp. 131. https://doi.org/10.1038/s41420-022-00930-9.
18. Mancino, Mario, et al. “The Neuronal Influence on Tumor Progression.” *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1816, no. 2, Dec. 2011, pp. 105–18. https://doi.org/10.1016/j.bbcan.2011.04.005.

19. Lan, Yu-Long, et al. “Progress in Cancer Neuroscience.” *MedComm*, vol. 4, no. 6, Dec. 2023, pp. e431. https://doi.org/10.1002/mco2.431.
20. Silverman, Deborah A., et al. “Cancer-Associated Neurogenesis and Nerve-Cancer Cross-Talk.” *Cancer Research*, vol. 81, no. 6, Mar. 2021, pp. 1431–40. https://doi.org/10.1158/0008-5472.CAN-20-2793.
21. Guo, Weinan, et al. “Signal Pathways of Melanoma and Targeted Therapy.” *Signal Transduction and Targeted Therapy*, vol. 6, no. 1, Dec. 2021, pp. 424. https://doi.org/10.1038/s41392-021-00827-6.
22. Barbosa, Inês A. M., et al. “Cancer Lineage-Specific Regulation of YAP Responsive Elements Revealed through Large-Scale Functional Epigenomic Screens.” *Nature Communications*, vol. 14, no. 1, July 2023, pp. 3907. https://doi.org/10.1038/s41467-023-39527-w.
23. Dehingia, Bondita, et al. “CTCF Shapes Chromatin Structure and Gene Expression in Health and Disease.” *EMBO Reports*, vol. 23, no. 9, Sept. 2022, pp. e55146. https://doi.org/10.15252/embr.202255146.
24. Cui, Yue, et al. “Advances in *Cis* -Element- and Natural Variation-Mediated Transcriptional Regulation and Applications in Gene Editing of Major Crops.” *Journal of Experimental Botany*, vol. 74, no. 18, Sept. 2023, pp. 5441–57. https://doi.org/10.1093/jxb/erad248.
25. Flitsch, Lea J., et al. “Transcription Factor-Based Fate Specification and Forward Programming for Neural Regeneration.” *Frontiers in Cellular Neuroscience*, vol. 14, May 2020, pp. 121. https://doi.org/10.3389/fncel.2020.00121.
26. Wang, Haofei, et al. “Direct Cell Reprogramming: Approaches, Mechanisms and Progress.” *Nature Reviews Molecular Cell Biology*, vol. 22, no. 6, June 2021, pp. 410–24. https://doi.org/10.1038/s41580-021-00335-z.
27. Kenny, Colin, et al. “TFAP2 Paralogs Facilitate Chromatin Access for MITF at Pigmentation and Cell Proliferation Genes.” *PLOS Genetics*, vol. 18, no. 5, May 2022, pp. e1010207. https://doi.org/10.1371/journal.pgen.1010207.
28. Lal, Geeta, et al. “Human Melanoma Cells Over-Express Extracellular Matrix 1 (ECM1) Which Is Regulated by TFAP2C.” *PLoS ONE*, vol. 8, no. 9, Sept. 2013, pp. e73953. https://doi.org/10.1371/journal.pone.0073953.
29. Kim, Wanyeon, et al. “TFAP2C-Mediated Upregulation of TGFBR1 Promotes Lung Tumorigenesis and Epithelial–Mesenchymal Transition.” *Experimental & Molecular Medicine*, vol. 48, no. 11, Nov. 2016, pp. e273–e273. https://doi.org/10.1038/emm.2016.125.

30. Rosenblum, Daniel, et al. “CRISPR-Cas9 Genome Editing Using Targeted Lipid Nanoparticles for Cancer Therapy.” *Science Advances*, vol. 6, no. 47, Nov. 2020, pp. eabc9450. https://doi.org/10.1126/sciadv.abc9450.
31. Zhang, Di, et al. “Enhancing CRISPR/Cas Gene Editing through Modulating Cellular Mechanical Properties for Cancer Therapy.” *Nature Nanotechnology*, vol. 17, no. 7, July 2022, pp. 777–87. https://doi.org/10.1038/s41565-022-01122-3.

TABLES AND FIGURES

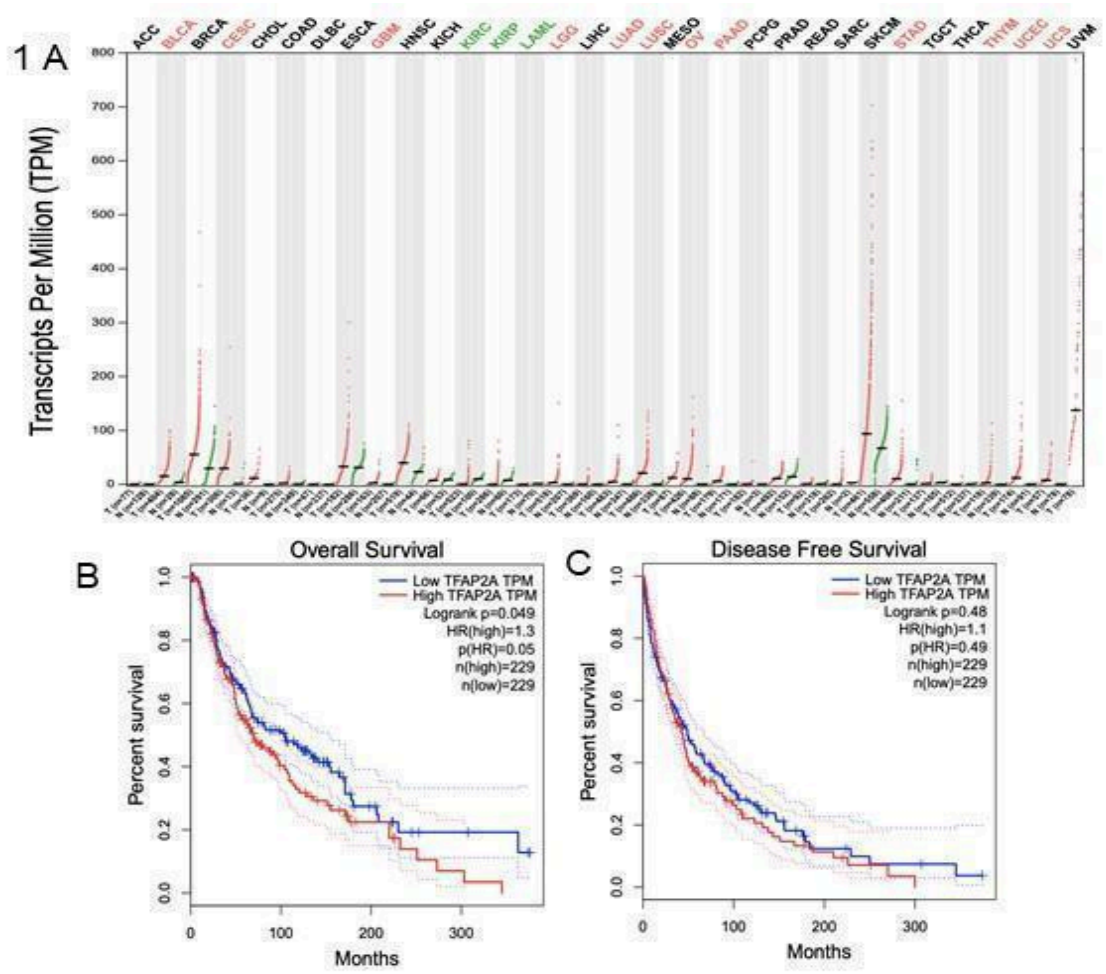

Figure 1. Differential expression for TFAP2A across cell lines and its overall and disease-free survival maps. GEPIA analysis shows that TFAP2A is the most critical isoform for targeting due to it having the largest differential expression (1A) and overall (1B) disease-free (1C) survival impact among the other TFAP2 isoforms.

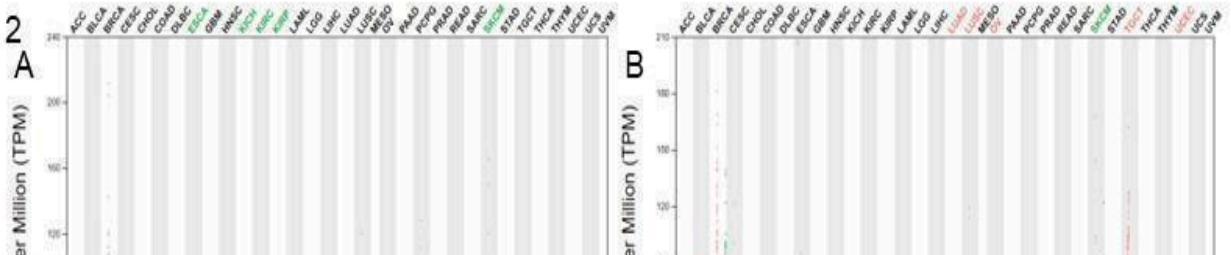

Figure 2. Differential expression of alternate isoforms across cell lines. Coupled GEPIA analysis alongside TFAP2A of TFAP2B, TFAP2C, and TFAP2E across cell lines can be compared to Figure 1 to show a lessened differential expression, making them a less than ideal target as compared to TFAP2A.

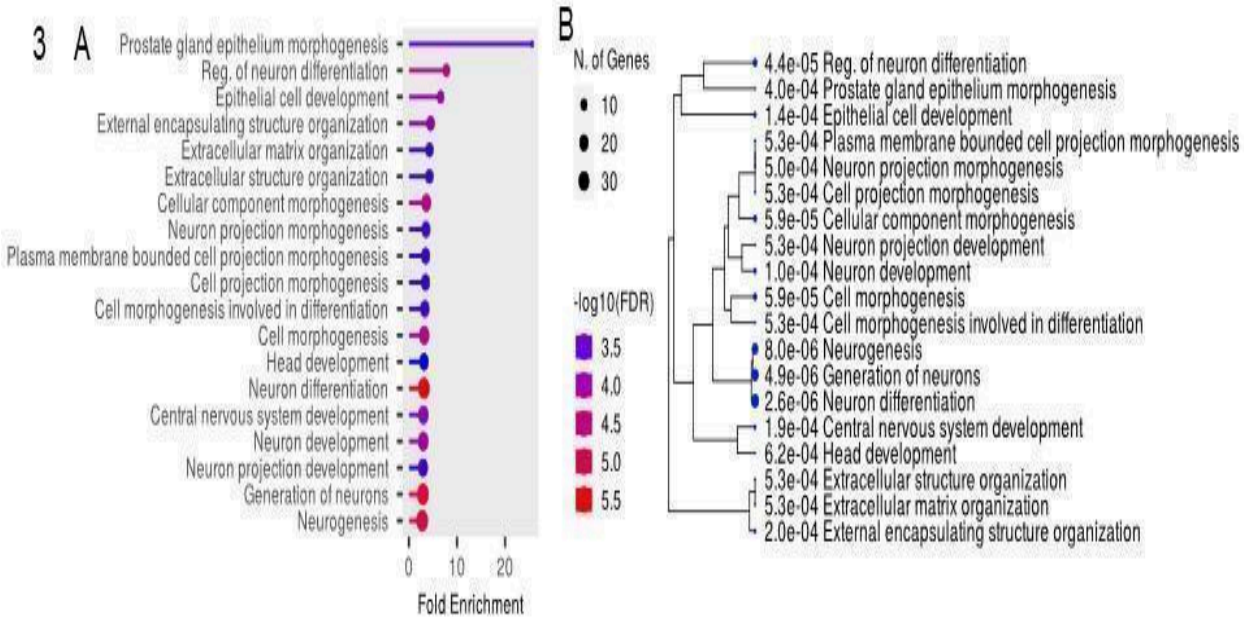

Figure 3. Gene ontology analysis. Pathway enrichment analysis (left) displaying down regulated genes after TFAP2 knockout and tree network analysis (right) showing all pathways showing neuronal differentiation related pathways.

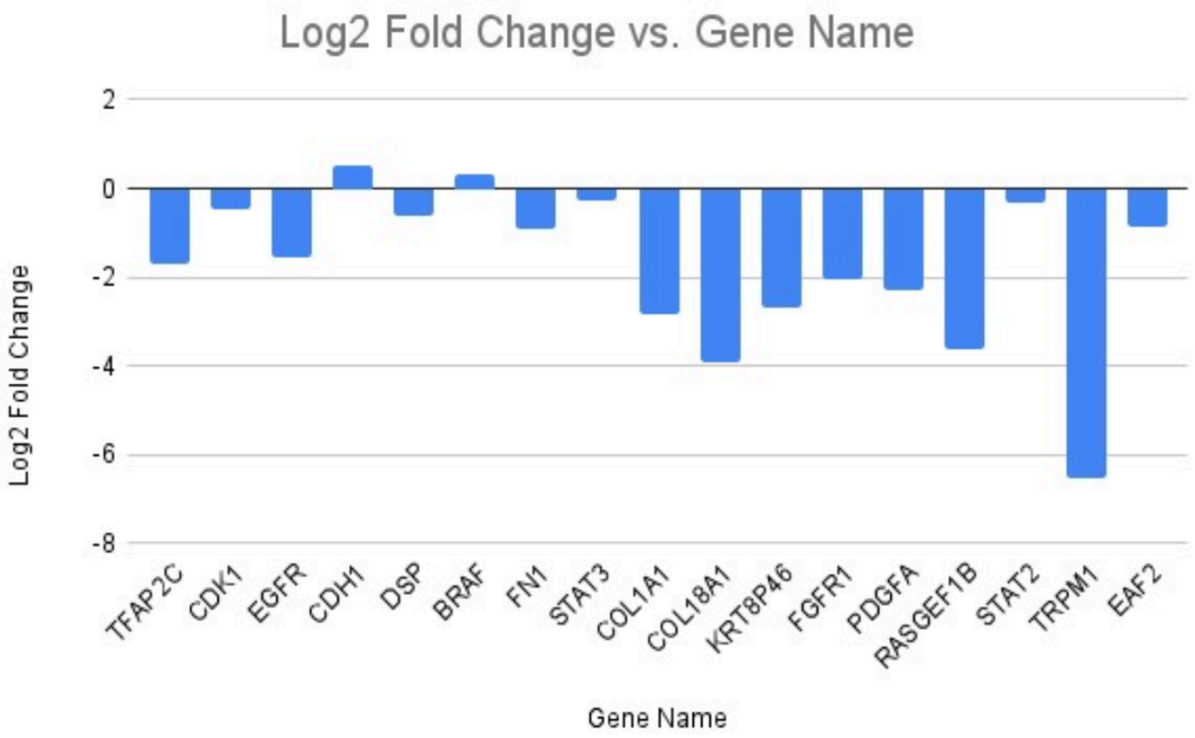

Figure 4: Log2 fold change vs gene name. A bar plot of gene downregulation. We observed after TFAP2 knockout the log2FC of our targeted gene list has drastically decreased. This suggests that TFAP2 can be a good target for CRISPR therapy.

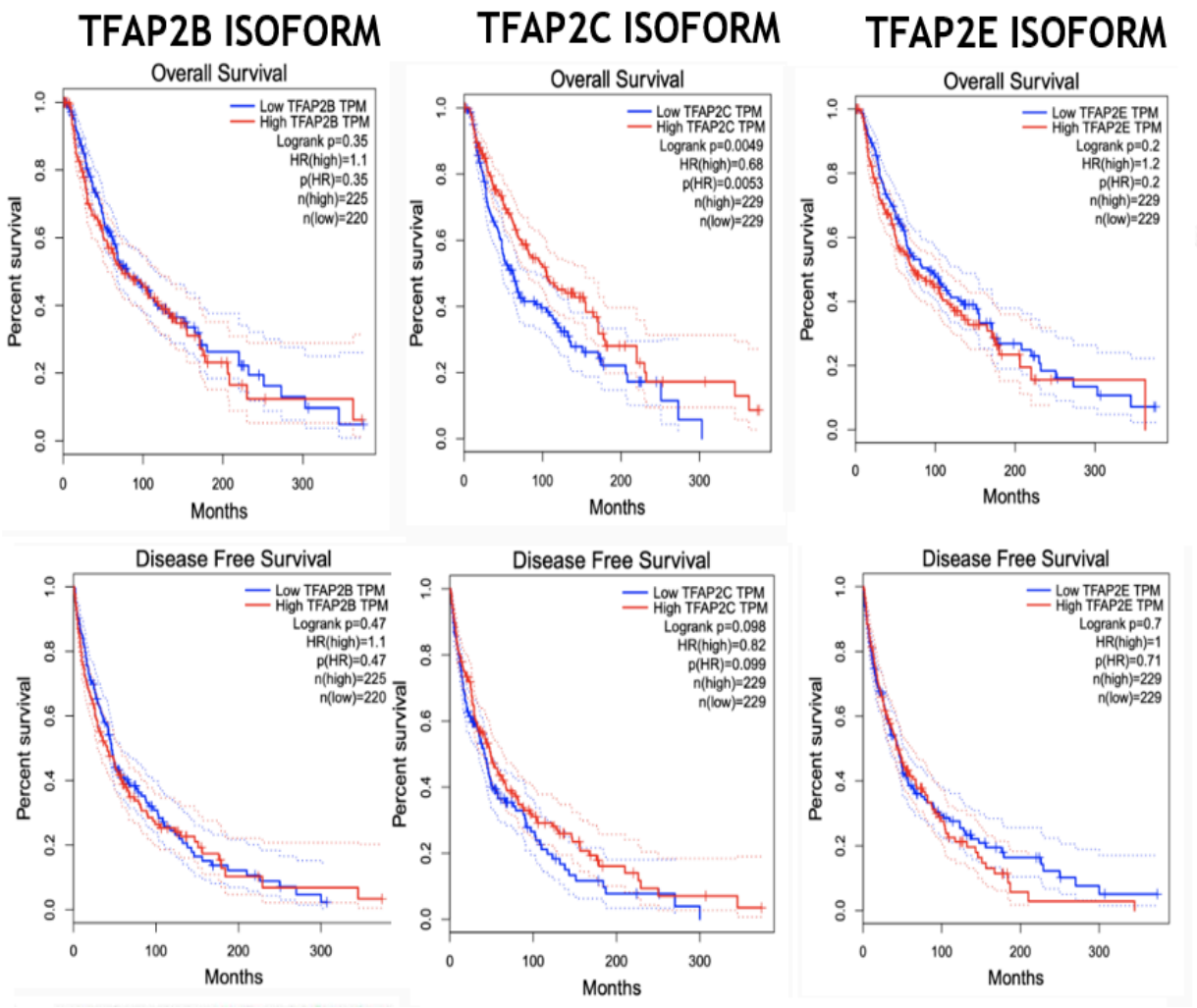

Figure 5: TFAP2 alternate isoform survival maps. The overall and disease-free survival across B, C and E isoforms as less steep and a lower hazard ratio point to less of a survival impact than TFAP2, with higher hazard ratio and steeper survival curves.

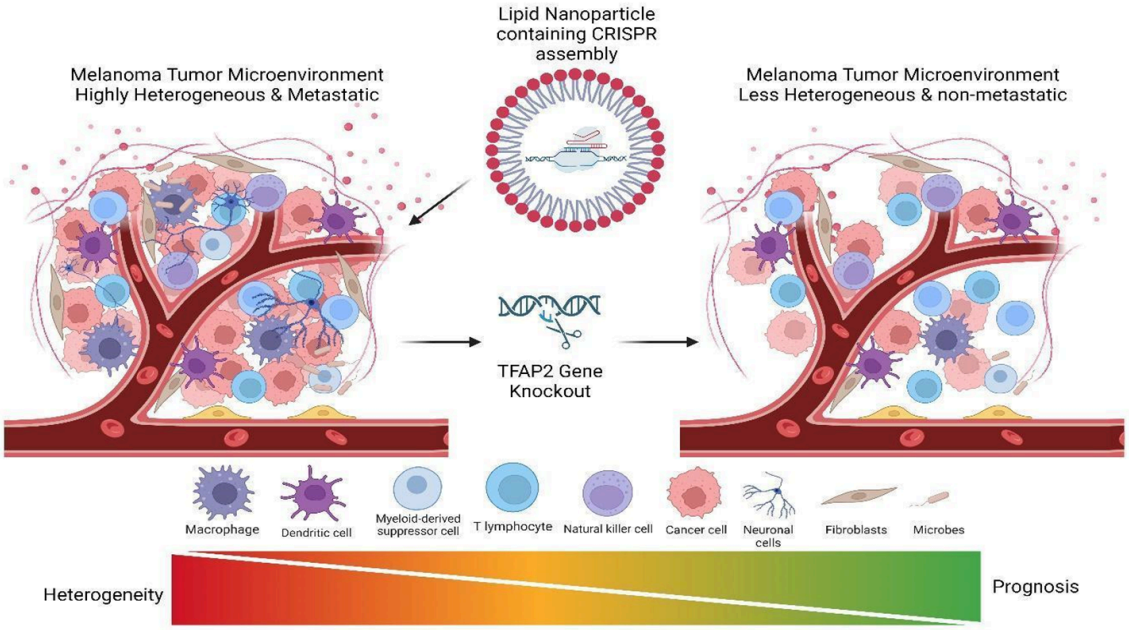

Figure 6. **Graphical abstract.** showing the overall effect of TFAP2 knockout on tumor heterogeneity in increasing prognosis.