AWARD NUMBER: W81XWH-21-1-0106

TITLE: Therapeutic Targeting of Nuclear Hormone Receptors in Neurofibromin/NF1-Depleted Breast Cancer

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REPORT DATE: April 2024

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
April 2024	Annual	15Mar2023-14Mar2024

4. TITLE AND SUBTITLE

Therapeutic Targeting of Nuclear Hormone Receptors in Neurofibromin/NF1-Depleted Breast Cancer

5a. CONTRACT NUMBER W81XWH-21-1-0106

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER 0011582061

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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U.S. Army Medical Research and Developmentl Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project centers on the NF1/neurofibromin tumor suppressor, which was best known as a GTPase Activating Protein (GAP) that represses Ras activity. We have recently shown that NF1 has a GAP-independent activity by functioning also as a transcriptional co-repressor for estrogen receptor α (ER) in ER^+ breast cancer. ER is structurally closely related to the androgen receptor (AR). In this multi-PI grant, we will investigate the hypothesis that We **hypothesized** that NF1, analogous to its role in ER regulation, is also an AR co-repressor. Our **objective** is to assess this hypothesis to explore and exploit the broader consequences of NF1 loss in breast cancer therapeutics. The first specific aim is to define the interactions between neurofibromin and AR by studying neurofibromin's role as an AR co-repressor. Our results showed that AR and NF1 can physically interact in a ligand-dependent manner. While NF1-silencing enhanced AR-dependent transcriptional activities, NF1 overexpression inhibited it. As a result, NF1-depleted AR^+ cancer cells can grow at suboptimal levels of AR agonists. The second specific aim is to assess how hyperactivated AR due to *NF1* loss impacts the treatment of breast cancer by pre-clinically modeling the effects of AR antagonists or SARMs (selective AR modulators). The results from this project period showed that NF1-depletion can affect the choices of anti-AR agents. Enzalutimde which does not have known agonist activity is a better drug to treat these tumors than bicalutamide. Further NF1 loss actives not only AR but also Ras. In support of this, adding a MEK inhibitor can enhance the efficacy of enzalutamide.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	10	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

The *NF1* gene encodes neurofibromin. The *NF1* gene is widely inactivated in cancer, and thus considered a key tumor suppressor gene. While NF1/neurofibromin is best known as a GTPase Activating Protein (GAP) that represses Ras activity, we have recently shown that it can also repress the transcriptional activity of the estrogen receptor α (ER) in ER^{+} breast cancer. This transcriptional activity is independent of GAP activity but dependent on conserved amino acid sequences that are responsible for ER binding. In a cancer database search, we found a loss of function mutation affecting one of the ER-binding sites in prostate cancer, in which the androgen receptor (AR) is a key drug target. AR and ER are highly structurally related, which led to our initial inquiry of whether AR and NF1 can similarly interact. We have thus obtained preliminary data supporting the **hypothesis** for this proposal that NF1 can also function as a transcriptional co-repressor for AR. The objective of this proposal is to assess the broader consequence of NF1 loss in breast cancer therapeutic. The specific aims are:

AIM 1: To define the interactions between neurofibromin and AR by studying neurofibromin's role as an AR co-repressor (primary responsibility of the initiating PI Eric Chang). We will measure:

(A) Ligand-dependent direct binding between neurofibromin and AR, using purified components.

(B) AR-dependent gene expression and AR recruitment to the chromatin as mediated by neurofibromin, using RNA-seq and ChIP-seq.

(C) AR ligand-mediated recruitment of neurofibromin to the chromatin by ChIP-seq.

AIM 2: To assess how hyperactivated AR due to *NF1* loss impacts the treatment of breast cancer by pre-clinically modeling the effects of AR antagonists or SARMs (primary responsibility of the partnering PI Matthew Ellis/Bora Lim).

(A) Measure *in vitro* cell growth/apoptosis upon neurofibromin-depletion in the presence of a SARM (ER^{+} models) or an AR antagonist (ER^{-} models); if successful, these compounds will be tested in combination with a MEKi (binimetinib).
(B) Measure treatment efficacies using xenograft models, leveraging our collection of several *NF1*$^{-}$ and AR^{+} PDX models as a prelude to potential clinical trials.

KEYWORDS

AI, Aromatase inhibitor
AR, Androgen receptor.
ARE, AR responsive element.
ChIP, Chromatin immunoprecipitation
Cut&Run: Cleavage Under Targets and Release Using Nuclease
Co-IP, co-immunoprecipitattion.
DCIS, ductal carcinoma *in situ*
DHT, Dihydrotestosterone
DOX, doxycycline.
E2, estrogen/estradiol
EMT, epithelial to mesenchymal transition
ER, estrogen receptor-α
EREs, Estrogen Response Elements
FS, frameshift
GAP, GTPase Activating Protein

IHC, immunohistochemistry
KI, knock-in
KM, Kaplan-Meier
KO, knock-out
MEKi, MEK inhibitor.
NF1, Neurofibromatosis type 1
NS, nonsense
PDX, patient-derived xenograft
SARM, Selective AR modulator
SERM, Selective ER modulator
TCGA, The Cancer Genome Atlas

ACCOMPLISHMENTS

AIM 1: To define the interactions between neurofibromin and AR by studying neurofibromin's role as an AR co-repressor. (Primary responsibility of Initiating PI: Chang)

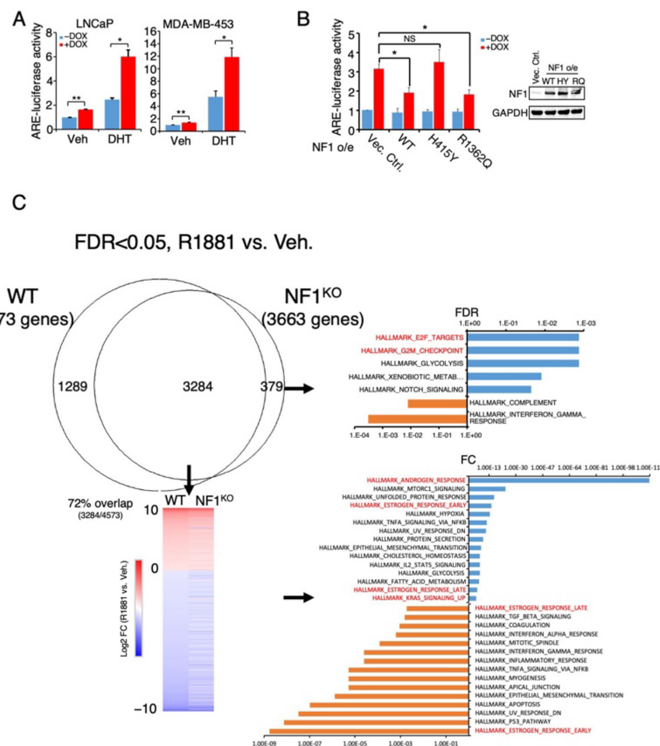

Fig. 1. NF1-depletion enhances AR transcriptional activity. (A) When NF1 expression was silenced by DOX, activity from an ARE-luc reporter increased in the presence of AR ligand DHT in two separate cell lines. (B) Wild type and various NF1 mutants were expressed to assess the ability to repress the enhanced ARE-luc activity (see main text), and the Western blot on the right showed all NF1 proteins examined were expressed at comparable levels. (C) Left: RNA-seq was performed on parental and *NF1* knockout (KO) cells with or without the AR agonist R1881. The Venn diagram shows that the expression of 3293 genes out of 4584 R1881-responsive genes, or 72%, was altered after NF1-depletion. The most enriched pathways common to both parental and KO cells and unique to KO cells were identified by over representation analysis.

(A) <u>Ligand-dependent direct binding between neurofibromin and AR, using purified components</u>. In the previous project cycle, we have completed this subaim showing that purified NF1 can bind purified AR in a ligand-dependent manner. These results support the hypothesis that their interaction is direct.

(B) <u>AR-dependent gene expression and AR recruitment to the chromatin as mediated by neurofibromin, using RNA-seq and ChIP-seq</u>. In the past project periods, we have used a luciferase reporter construct to show that when *NF1* was knocked down in breast cancer MDA-MB-435 cells, the reporter activities increased substantially (Fig. 1A). Conversely, in this project period, we further showed that wild type NF1, when ectopically expressed, reduced the reporter activities (Fig. 1B). In contrast, the NF1-H415Y mutant which lacks AR binding capability did not. Furthermore, AR repression by NF1 is independent of NF1's GAP activity because the NF1 GAP mutant NF1-R1362Q worked as well as wild type NF1 in repressing ARE-luc activity.

In the previous project period, we have begun using RNA-seq to show that NF1-depletion enhanced endogenous AR ligand-dependent gene expression. In this project period, we have polished this line of studies by adding gene set enrichment analysis (Fig. 1C) to show that the changes in gene expression affects androgen signaling pathway as expected. To a lesser degree,

RAS activation was also detected. These results are consistent with the model that NF1 is a dual repressor inhibiting both RAS and AR. This figure is now ready for publication.

Finally, as reported last year, by ChIP-qPCR, we determined that the changes in gene expression is caused by more AR recruited to the gene promoters.

(C) AR ligand-mediated recruitment of neurofibromin to the chromatin by ChIP-seq. In the past project periods, we performed ChIP-qPCR and found that NF1 was recruited to the promoter regions in the presence of the AR antagonist RU486, which agreed with the hypothesis that NF1 is a co-repressor.

To detect NF1 association with chromatin in an un-biased manner and to identify all the genes regulated by NF1, we attempted an approach known as Cut&Run (Cleavage Under Targets and Release Using Nuclease). This approach is generally considered to be much more sensitive than conventional ChIP-seq to address the concern that NF1 may be weakly and indirectly associated with DNA as a transcriptional co-repressor. We have performed the IP and awaiting the sequencing results to come back.

AIM 2: To assess how hyperactivated AR due to *NF1* loss impacts the treatment of breast cancer by pre-clinically modeling the effects of AR antagonists or SARMs (primary responsibility of the partnering PI Dr. Alastair Thompson*).

*In last project period, Dr. Bora Lim took over the responsibility as the partnering PI after the original Partnering PI, Matthew Ellis, departed BCM. Later Dr. Lim accepted a position at MD Anderson Cancer Center, and Dr. Alastair Thompson kindly took over from her the leadership.

(A) Measure *in vitro* cell growth/apoptosis upon neurofibromin-depletion in the presence of a SARM (ER^+ models) or an AR antagonist (ER^- models); if successful, these compounds will be tested in combination with a MEKi (binimetinib).

This line of study so far has been focused mostly on cancer cells whose growth is strongly AR dependent. This includes the AR^+ ER^- breast cancer cell line MDA-MB-453 breast cancer cell lines and several prostate cancer cell lines (e.g., LNCaP and VCaP). In the previous project periods, we showed that when NF1 was depleted from these cell lines, the resulting cells became much more sensitive to not only AR agonists, such as R1881 and DHT, but also to RU486, which can inhibit AR. These observations are consistent with the concept that NF1 is an AR co-repressor.

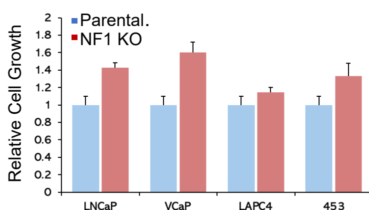

Fig. 2. NF1-depleted AR^+ cancer cells can grow in the absence of added androgens in the culture media. Several parental and NF1-KO cell lines were seeded in culture media containing charcoal-stripped serum, and cell growth was measured by MTS assay 7 days later. The difference between NF1+ and NF1-KO cells are all statistically significant by t-test.

In this project period, we more carefully examined the number of cells seeded in vehicle or increasing concentration of the AR agonist R1881 and found at NF1-depleted cells grew better in vehicle treated cells (Fig. 2). This observation supports the concept that NF1-depletion can promote castration-resistance. Enzalutamide is a new generation AR antagonist used to treat castration-resistant tumors. In the last project period, we showed that when NF1 was depleted from these cells, the resulting cells grew better than the parent cells *in vitro*. This raises the possibility that NF1-depleted AR^+ cancer patients should not be treated by enzalutamide either. In this project period, we have begun to validate this *in vivo* using xenograft models see Aim 2B below.

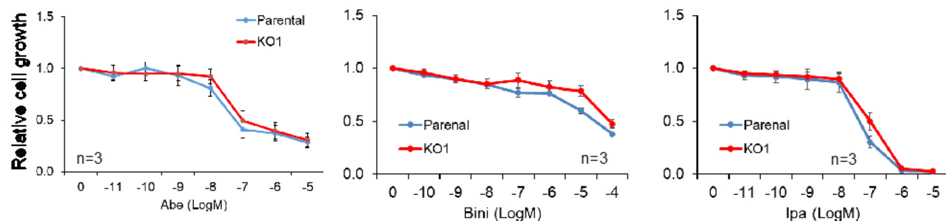

Fig. 3. Enzalutamide plus binimetinib, ipatasertib, or abemaciclib did not selectively inhibit the growth of NF1-depleted AR⁺ cancer cells. Parental and NF1-KO cells were seeded in the presence of enzalutamide to which indicated drugs were later added in increasing amounts. Cell growth was measure by MTT assay 7 days later.

While NF1-depletion promotes AR activity, the RAS pathways are expected to become active too, because of the loss of GAP activity. We have thus begun to investigate the use of MEK inhibitor (binimetinib) and AKT inhibitor (ipatasertib) in combination with endocrine therapy to treat NF1-depleted AR^+ cancer. Furthermore, we also investigated adding abemaciclib, a CDK4/6 inhibitor, to assess *in vitro* the best combination therapy to treat NF1-depleted cancer cells. Below are our findings:

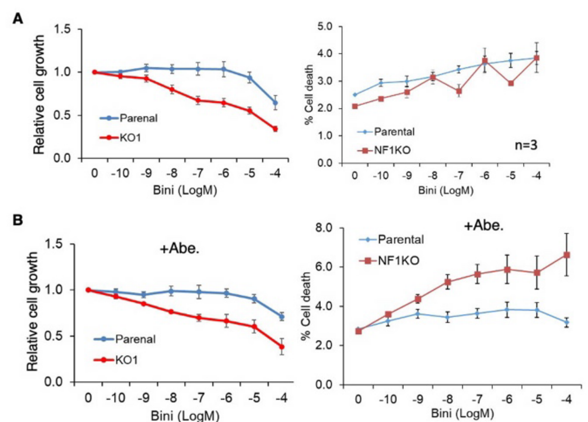

Fig. 4. Androgen deprivation is a more effective form of endocrine therapy when combined with kinase inhibitors in the treatment of NF1-depleted AR^+ cancer cells. (A) Parental and NF1-KO cells were seeded in androgen-deprived medium (medium containing charcoal-stripped serum), to which various concentrations of binimetinib was added. Seven days later, cell growth was measured by MTS while cell death by CellTox. (B) The same as in (A) except the medium also contained 10 nM abemaciclib (Abe.).

We first combine the endocrine agent enzalutimide with either binimetinib or ipatasertib and found that these combinations did not selectively inhibit the growth of NF1-depleted cells *in vitro* — both $NF1^+$ and $NF1^-$ cells show the same degrees of growth inhibition (Fig. 3). In contrast, when castration (seeding the cells in media containing charcoal-stripped serum) was used as the endocrine approach, combination therapy with binimetinib can more effectively inhibit cell growth of NF1-depleted AR^+ cell lines (Fig 4A). This combination does not induce cell death (Fig. 4A); however, cell death was substantially induced when abemaciclib was also added (Fig. 4B). Finally we found that the combination of abemaciclib + binimetinib or ipatasertib can equally induce cell death without androgen deprivation (Fig. 5).

(B) <u>Measure treatment efficacies using xenograft models</u>.

Our *in vitro* data showed that AR^+ NF1-depleted cancer cells are resistant to enzalutamide. We have begun to investigate this *in vivo* using xenograft models. The results are encouraging (Fig. 6), although we are still waiting for tumors to emerge in more mice before the treatment.

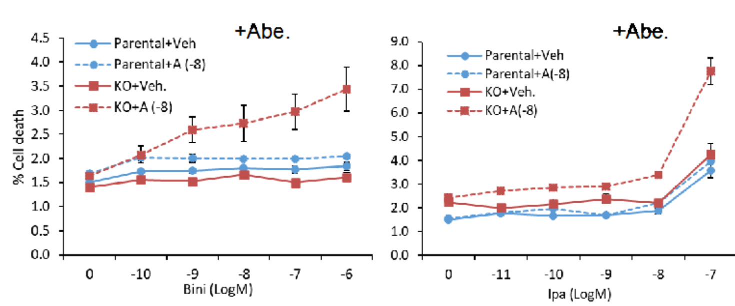

Fig.5. Abemaciclib plus either binimetinib or ipatasertib can efficiently induced the death of NF1-depleted AR+ cancer cells without androgen deprivation. Cells were seeded in regular medium containing 10 nM abemaciclib to which binimetinib or ipatasertib was added. The growth and death of cells were measured as those in Fig. 4.

IMPACT

We recently published an IHC assay that directly measures NF1 protein levels in patient FFPE samples (PMID: 37501682). Our findings revealed that over 21% of an early-stage ER+ breast tumor cohort had lost NF1, which led to aromatase inhibition resistance. Consequently, NF1-loss is a common occurence in ER^+ breast cancer, significantly impacting patient outcomes. Interestingly, AR expression is even more prevalent than ER expression—90% of breast tumors are AR^+. As demonstrated by data from this

study, it is very clear that NF1-loss will also activate AR, affecting not only ER^+ breast cancer but also prostate cancer. Our study has now entered the phase of defining the most effective treatment strategy for $NF1^-$ AR^+ cancer using FDA-approved agents. There is a possibility that we may discovery a treatment approach that can be readily implemented in clinical practice.

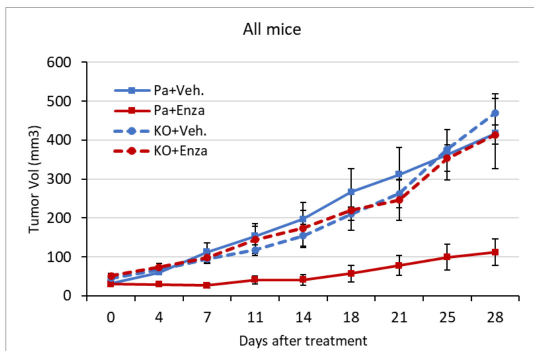

Fig. 6. NF1-depletion promotes resistance to enzalutamide *in vivo*. Parental and NF1-KO AR^+ cancer cells (LNCaP) were transplanted to nude mice which were then treated with enzalutamide or vehicle. Tumor volume was measured twice per week.

CHANGES/PROBLEMS

After the departure of the original partnering PI Dr. Matthew Ellis left BCM, Dr. Bora Lim kindly took over. However, Dr. Lim has since moved to another institution; therefore, Dr. Alastair Thompson a physician scientist stepped in as the current partnering PI. Dr. Thompson is the chief of breast surgery Michael E. DeBakey Department of Surgery, Division of Surgical Oncology, and co-director of the Lester and Sue Smith Breast Center, part of the Dan L Duncan Comprehensive Cancer Center. Dr. Thompson has worked closely with Dr. Chang on many other projects before. For example, Dr. Thompson is a world leader in the study of DCIS (ductal carcinoma *in situ*), a key breast cancer premalignancy. With his help, Dr. Chang has recently awarded a DoD grant studying DCIS. With Dr. Thompson's leadership, the *in vivo* study is going well.

PRODUCTS

Clinical trial

The concept that NF1 as a co-repressor for nuclear receptors has resulted in a clinical trial by first centering on ER^+ breast cancer. This study, EAY191-N2, has opened as of March 2023, as one of the first 4 studies as part of the National Cancer Institute CTEP ComboMATCH initiatives (NCT05554354). After reviewing a few patient candidates that we thought would be eligible for the trial, we realized the eligibility was too strict.

First, the study, following the ComboMATCH policy, initially exclude the use of liquid biopsy to determine the *NF1* mutations. Second, the study had limited language regarding the eligible types *NF1* mutations. However, after extensive discussions with the leadership of the NCI CTEP biomarker and ComboMATCH team, we successfully obtained approval for a second amendment (which occurred during the second week of April 2024). This amendment now introduces new criteria for eligible *NF1* mutations.

Specifically, the updated amendment broadens the molecular eligibility criteria from '*NF1* non-sense or frameshift mutation' to '*NF1* molecular alterations that are considered to cause functional loss of NF1 protein.' As a result, the eligibility criteria have been expanded to encompass a wider range of *NF1* mutations. The "MatchBox" has been updated accordingly to prepare for the new molecular biomarker-based pipeline, which will assess eligibility decision based on these revised criteria. In addition, the protocol has been presented on the NCI's YouTube channel and discussed during the Spring meeting of NRG.

Publication

Besides studying NF1 in the context of AR regulation, this project has also assisted in the publication of proteomic methods in the detection of NF1 at the protein levels. This is a very important project because

assessing NF1 status by DNA sequencing, as it is commonly used, greatly underestimates the number of NF1 loss-of-function tumors, making it difficult to deliver targeted therapy to the right patients:

Kim BJ, Zheng ZY, Lei JT, Holt MV, Chen A, Peng J, Fandino D, Singh P, Kennedy H, Dou Y, Chica-Parrado MDR, Bikorimana E, Ye D, Wang Y, Hanker AB, Mohamed N, Hilsenbeck SG, Lim B, Asirvatham JR, Sreekumar A, Zhang B, Miles G, Anurag M, Ellis MJ, **Chang EC**. 2023. Proteogenomic Approaches for the Identification of NF1/Neurofibromin-depleted Estrogen Receptor-positive Breast Cancers for Targeted Treatment. Cancer Res. Commun. 3:1366-1377. PMCID:PMC10370361, PMID:37501682.

NF1 loss leads to RAS activation. In a related study, we focuseed on sotorasib, which is a new line of therapy to specifically inhibit the activity of the oncogenic KRAS-G12C. A manuscript resulted from this collaboration has been published in *Science*:

Xiangdong Lv; Xuan Lu; Jin Cao; Qin Luo; Yao Ding; Fanglue Peng; Doug W Chan; Xiaoran Wang; Sara R. Savage; Sufeng Mao; Jingjing Yu; Fei Peng; Yan Liang; Huan Meng; Laure Maneix; Yumin Han; Yiwen Chen; Wantong Yao; **Eric C. Chang**; Andre Catic; Xia Lin; George Miles; Pengxiang Huang; Zheng Sun; Huamin Wang; Qizhi Cathy Yao; Bing Zhang; Bert W O'Malley; Mattew J Ellis; Mothaffar F Rimawi; Haoqiang Ying; Xi Chen. 2023. Modulation of the proteostasis network promotes tumor resistance to oncogenic *KRAS* inhibitors. 381: eabn4180. *Science.* PMID: 37676964. PMCID: PMC10720158.

Finally, in a separate study, we found that NF1-loss can promote metastasis, an area of high priority area of the DoD. We have thus collaborated with colleagues to study metastatic tumors by single cell technologies. The following two manuscripts have been submitted for publication:

Qian Zhu, Akhila Balasubramanian, Jaya Ruth Asirvatham, Danthasinghe Waduge Badrajee Piyarathna, Jaspreet Kaur, Nada Mohamed, Ling Wu, Megha Chatterjee, Stacy Wang, Niloufar Pourfarrokh, Uttam Rasaily, Yitian Xu, Junjun Zheng, Deborah Jebakumar, Arundathi Rao, Shu-Hsia Chen, Yi Li, **Eric Chang**, Xiaoxian Li, Ritu Aneja, Xiang H-F Zhang, Arun Sreekumar. 2024. Integrative spatial omics reveals distinct tumor-promoting multicellular niches and immunosuppressive mechanisms in African American and European American patients with TNBC. Submitted to *Nature Genetics.*

Arun Sreekumar, qian zhu, Akhila Balasubramanian, Jaya Asirvatham, Danthasinghe Waduge Badrajee Piyarathna, Jaspreet Kaur, Nada Mohamed, Ling Wu, Megha Chatterjee, Stacy Wang, Niloufar Pourfarrokh, Uttam Rasaily, Yitian Xu, Junjun Zheng, Deborah Jebakumar, Arundathi Rao, Shu-Hsia Chen, Yi Li, **Eric Chang**, Xiaoxian Li, Ritu Aneja, and Xiang Zhang. 2024. Integrative spatial omics reveals distinct tumor-promoting multicellular niches and immunosuppressive mechanisms in African American and European American patients with TNBC. Submitted to *Cancer Discovery.*

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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Project Role: PI
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Nearest person month worked: 4.2CM
Contribution to Project: Design and execute all the studies in this project and will write the paper.

Name: Zeyi Zheng
Project Role: Instructor
ORCID ID: 0000-0001-6536-4874
Nearest person month worked: 6.2CM
Contribution to Project: Assist Dr. Chang in the design and execution of all the studies in this project and supervise Ms. Kenney.

Name: Hilda Kennedy
Project Role: Research Technician
ORCID ID: N/A
Nearest person month worked: 6.2CM
Contribution to Project: Provide technical support on all projects.

Name: Matthew Baik
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ORCID ID: N/A
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Contribution to Project: Perform all described molecular studies.

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Nearest person month worked: 1.75CM
Contribution to Project: Perform in vitro studies and help organize the lab.