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Nothing to report

14. ABSTRACT

NF1 function is classically altered by gene deletion in breast cancer. An alternative-but-equally-important mechanism of NF1 deficiency is defective mRNA processing whereby mutant mRNA transcripts directly or indirectly affect the stability of wild type transcripts. This delicate balance of mutant versus wild type transcript abundance can dramatically affect protein synthesis and, ultimately, RAS signaling fates. In cancer, dysregulation of alternative splicing promotes malignant progression and therapeutic resistance by altering the expression and function of tumor suppressors and oncogenes. Little is known about NF1-related mRNA processing or how alternative transcripts affect NF-related phenotypes such as breast cancer. Our goal is to define the genetic and isoform changes in NF1 that occur in breast cancer with the ultimate goal of identifying prognostic biomarkers and targeted therapeutic strategies for both female and male NF patients with breast cancer. Our hypothesis is that alternative RNA splicing of NF1 abrogates NF1 gene function and RAS regulation to promote breast cancer progression and therapeutic resistance. Our experimental approaches leverage innovative sequencing methods, our established rat model of Nf1-deficient breast cancer, and comprehensive analysis of breast cancer datasets. The results of these studies will provide insight into the

15. SUBJECT TERMS
Neurofibromatosis Type I, breast cancer, alternative isoforms

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1. Introduction

NF1 function is classically altered by gene deletion in breast cancer. An alternative-but-equally-important mechanism of *NF1* deficiency is defective mRNA processing whereby mutant mRNA transcripts directly or indirectly affect the stability of wild type transcripts. This delicate balance of mutant versus wild type transcript abundance can dramatically affect protein synthesis and, ultimately, RAS signaling fates. In cancer, dysregulation of alternative splicing promotes malignant progression and therapeutic resistance by altering the expression and function of tumor suppressors and oncogenes. Little is known about *NF1*-related mRNA processing or how alternative transcripts affect NF-related phenotypes such as breast cancer. Our *goal* is to define the genetic and isoform changes in *NF1* that occur in breast cancer with the ultimate goal of identifying prognostic biomarkers and targeted therapeutic strategies for *both female and male* NF patients with breast cancer. Our *hypothesis* is that alternative RNA splicing of *NF1* abrogates *NF1* gene function and RAS regulation to promote breast cancer progression and therapeutic resistance. Our experimental approaches also leverage innovative gene and RNA sequencing methods, as well as our established rat model of *Nf1*-deficient breast cancer. The results of these studies will provide insight into the mechanisms whereby alternative *NF1* RNA isoforms and mRNA processing promote breast cancer. Moreover, the results of this study will reveal RNA isoform signatures of *NF1* deficiency that could serve as diagnostic or prognostic markers for both NF1-related and sporadic breast cancer.

2. Keywords

Neurofibromatosis Type 1, NF1, alternative isoforms, breast cancer, mRNA splicing

3. Accomplishments

See following pages 5-10

What were the major goals of the project?

Tasks and Milestones Achieved	**Timeline (Months)**
Submit for human subjects review and approval for work proposed in Aim 3.	Completed
Specific Aim 1: Determine *Nf1* isoform expression during *Nf1*-deficient breast cancer progression	
Subtask 1: Perform long-read RNA sequencing on Nanopore MinION	Completed
Subtask 2: Perform short-read RNAseq with Illumina platform	Completed
Subtask 3: Align to reference transcriptome, reassemble the transcriptome, identify RNA structural variants.	Completed
Subtask 4: Realign the direct RNA to the reassembled transcriptome. Reads will be grouped into isoforms based on exons/intron composition.	Completed
Specific Aim 2: Determine *Nf1* isoform expression in endocrine-resistant *Nf1*-deficient breast cancers.	
Subtask 1: Perform long-read RNA sequencing on Nanopore MinION	Completed
Subtask 2: Perform short-read RNAseq with Illumina platform	Completed
Subtask 3: Align to reference transcriptome, reassemble the transcriptome, identify RNA structural variants.	Completed
Subtask 4: Realign the direct RNA to the reassembled transcriptome. Reads will be grouped into isoforms based on exons/intron composition.	In progress
Specific Aim 3: Identify NF1 isoform expression in human breast cancers	
Subtask 1: Bioinformatic analysis of *NF1* isoform expression in METABRIC human breast cancer database.	Completed/In progress
Subtask 2: Bioinformatic analysis of *NF1* isoform expression in TCGA human breast cancer database.	Completed
Subtask 3: Determine correlation of alternative *NF1* isoform expression with survival, recurrence, and subtype.	Completed
Subtask 4: Perform WGCNA and unsupervised clustering to uncover connections between *NF1* status, RAS signaling, and ER signaling.	In progress
Subtask 5: Perform RNAseq on 20 ER+/PR+/HER2- and 20 ER-/PR-/HER2- breast cancer. These tissues were previously obtained under an approved IRB.	In progress
Subtask 6: Validate NF1 isoform expression in tissues assessed in Subtask 5.	In progress

What was accomplished under these goals?

Identification of a NF1 transcript variant with altered nuclear localization in breast cancer

To investigate whether specific NF1 transcript variants mimic the detrimental effects *NF1* alterations have on breast cancer disease outcome, we utilized the TCGA SpliceSeq database to identify splicing events of *NF1* in breast cancer samples from the TCGA breast cancer dataset. Among the detected *NF1* splicing events was exon 31 which is well known for its location in neurofibromin's gap related domain (GRD), affects neurofibromin's GTPase activating protein (GAP) function, and distinguishes NF1 type I from type II transcripts. Another transcript variant present in primary breast cancers contained the splicing event of exon 52, coding for the nuclear localization sequence (NLS) of neurofibromin (Figure 1A). Even though previous studies have suggested nuclear localization of neurofibromin could serve an important and has been shown to act as a co-repressor of estrogen receptor's transcriptional function in the nucleus, but the impact of the neurofibromin nuclear localization is poorly understood. Transcript variants with exon 52 spliced out (ΔNLS) prevent nuclear localization of neurofibromin. This *NF1* ΔNLS transcript variant is highly expressed relative to other *NF1* transcript variants in adult liver, lung, placenta, kidney, and skeletal muscle in adult humans. Unlike exon 31 splicing, which was detected in every tumor sample, expression of the ΔNLS splicing event occurred in only 21.3% (233/1095) of primary tumors and 48.6% (37/76) of normal adjacent tissue with varying percent spliced in (PSI) values (Figure 1B). We next filtered for tumor samples that contained clinical data and mutational status for established breast cancer predictors (Table 1) and performed PCA analysis on primary tumor samples to determine any association to tumors containing ΔNLS transcript expression. PCA analysis demonstrated that

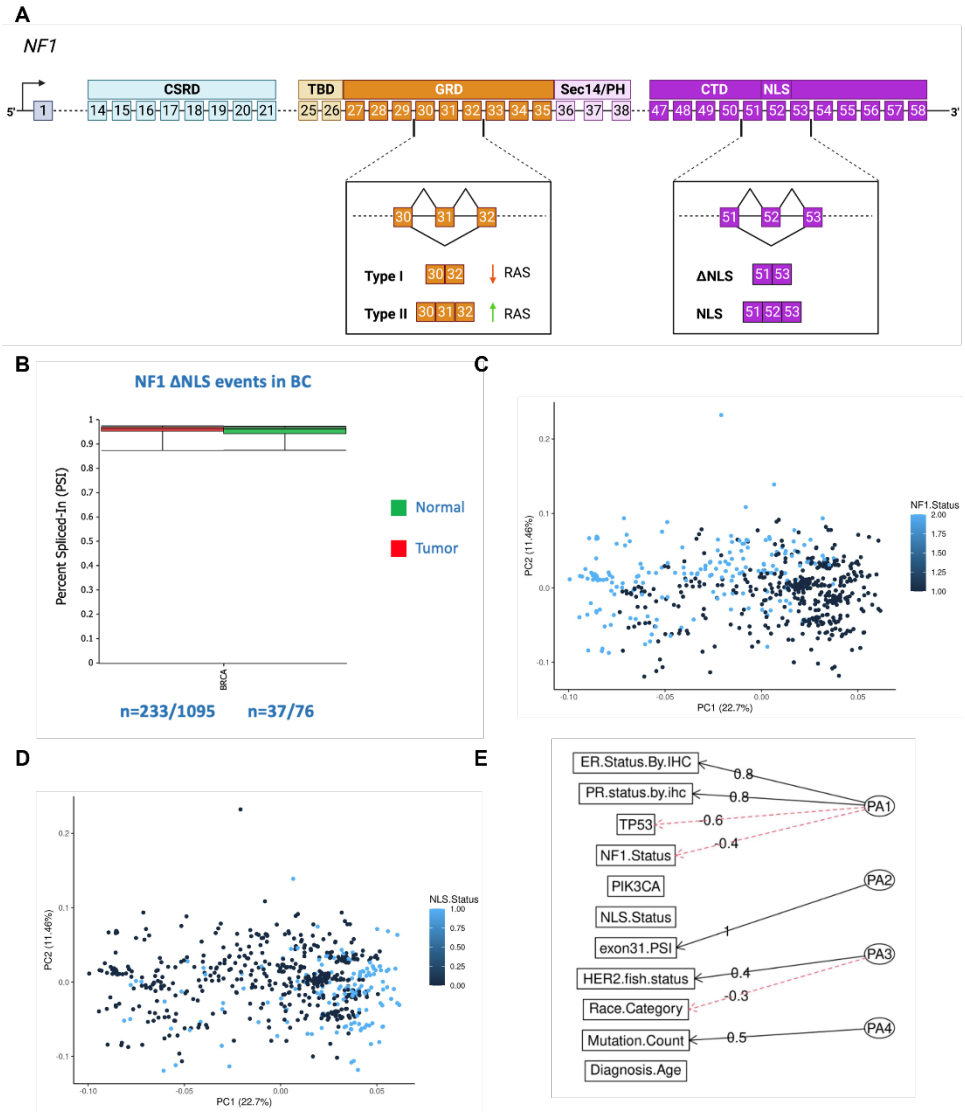

Figure 1: NF1 ΔNLS expressing tumors are a unique breast cancer subset. A) Schematic of *NF1's* coding exons and commonly spliced exons for RAS regulation in orange and nuclear localization sequence (NLS) in purple. B) Percent spliced in (PSI) of NLS exon (exon 52) in primary tumor samples and normal adjacent tissue samples. C and D) PCA plots showing NF1 NLS status is mutually exclusive of NF1 alteration status (NLS status: 1= ΔNLS expression, 0= no ΔNLS expression) (NF1 Status: 1= NF1 Diploid, 2= NF1 Hetloss). E) Factor analysis of clinical predictors for breast cancer and *NF1* NLS status.

ΔNLS expressing tumors do not serve as an accompanying event to established prognostic markers (data not shown). Interestingly, only 19 tumors harbored both *NF1* hetloss status and ΔNLS expression suggesting that *NF1* status and ΔNLS expression are present as mutually exclusive events in primary breasts cancers (Figure 1C and 1D). To further validate that ΔNLS expressing tumors are not influenced by confounding factors, we ran a factor analysis on the same variables used in our PCA analysis. Factor analysis shows that ΔNLS expressing tumors do not associate with factors that cause observed variables suggesting that ΔNLS impact on survival is independent of known confounding factors (Figure 1E).

Stratified by NLS Status

	UNSPLICED (N=523)	SPLICED (N=148)	Overall (N=671)
PIK3CA			
NO	362 (69.2%)	91 (61.5%)	453 (67.5%)
YES	161 (30.8%)	57 (38.5%)	218 (32.5%)
TP53			
NO	327 (62.5%)	124 (83.8%)	451 (67.2%)
YES	196 (37.5%)	24 (16.2%)	220 (32.8%)
Race.Category			
ASIAN	45 (8.6%)	7 (4.7%)	52 (7.7%)
BLACK OR AFRICAN AMERICAN	57 (10.9%)	18 (12.2%)	75 (11.2%)
WHITE	421 (80.5%)	123 (83.1%)	544 (81.1%)
ER.Status.By.IHC			
0	141 (27.0%)	19 (12.8%)	160 (23.8%)
1	382 (73.0%)	129 (87.2%)	511 (76.2%)
PR.status.by.ihc			
0	194 (37.1%)	35 (23.6%)	229 (34.1%)
1	329 (62.9%)	113 (76.4%)	442 (65.9%)
Diagnosis.Age			
Mean (SD)	57.8 (12.6)	58.4 (13.9)	57.9 (12.9)
Median [Min, Max]	58.0 [28.0, 90.0]	59.0 [26.0, 90.0]	58.0 [26.0, 90.0]
Mutation.Count			
Mean (SD)	63.2 (209)	71.6 (298)	65.0 (231)
Median [Min, Max]	31.0 [1.00, 4280]	25.0 [3.00, 3430]	30.0 [1.00, 4280]
NLS.Status			
UNSPLICED	523 (100%)	0 (0%)	523 (77.9%)
SPLICED	0 (0%)	148 (100%)	148 (22.1%)
NF1.Status			
DIPLOID	340 (65.0%)	115 (77.7%)	455 (67.8%)
HETLOSS	183 (35.0%)	33 (22.3%)	216 (32.2%)

Table 1: Confounding factors that contribute to breast cancer prognosis stratified by *NF1* NLS status.

ΔNLS expressing breast tumors have decreased overall survival compared to non-ΔNLS expressing breast tumors

To determine if ΔNLS transcripts expression can be correlated with impact on breast cancer survival like *NF1* indels, we took our filtered samples (Table 1) and performed a Cox proportional-hazard (CPH) model to identify variables impacting overall survival. Our CPH model shows that the age of diagnosis and mutation count are the strongest contributors to impact on overall survival and ΔNLS expressing tumors is just on the cusp of statistically impacting overall survival (data not shown). Kaplan-Meier curve of ΔNLS expressing tumors (spliced) compared to non-ΔNLS expression tumors (unspliced) highlights decreased overall survival with separation of the spliced group occurring around sixty months, or five years, from diagnosis (Figure 2A). Interestingly, endocrine resistance in ER+ breast cancers occur around five years after diagnosis and begin to behave like TNBCs. This suggests that ΔNLS expressing tumors may correlate with endocrine resistant ER+ breast cancers or TNBCs. To test if ΔNLS expressing tumors have an impact on ER+ breast cancer survival, we filtered for samples with a PAM50 subtype and ran a CPH model to determine the effect of ΔNLS expression on overall survival among breast cancer subtypes. Kaplan-Meier curve and CPH model show overall survival is significantly impacted by ΔNLS expression (p-value = 0.044) in breast cancers with a PAM50 subtype (Figure 2B and Table 2). Like our first filtering process, we see separation in survival occur around five years in ΔNLS expressing tumor regardless of PAM50 subtype. We next separated Kaplan-Meier curves into each PAM50 subtype and saw no striking differences in TNBC or HER2-enriched subtypes. However, in ER+ subtypes (Luminal A

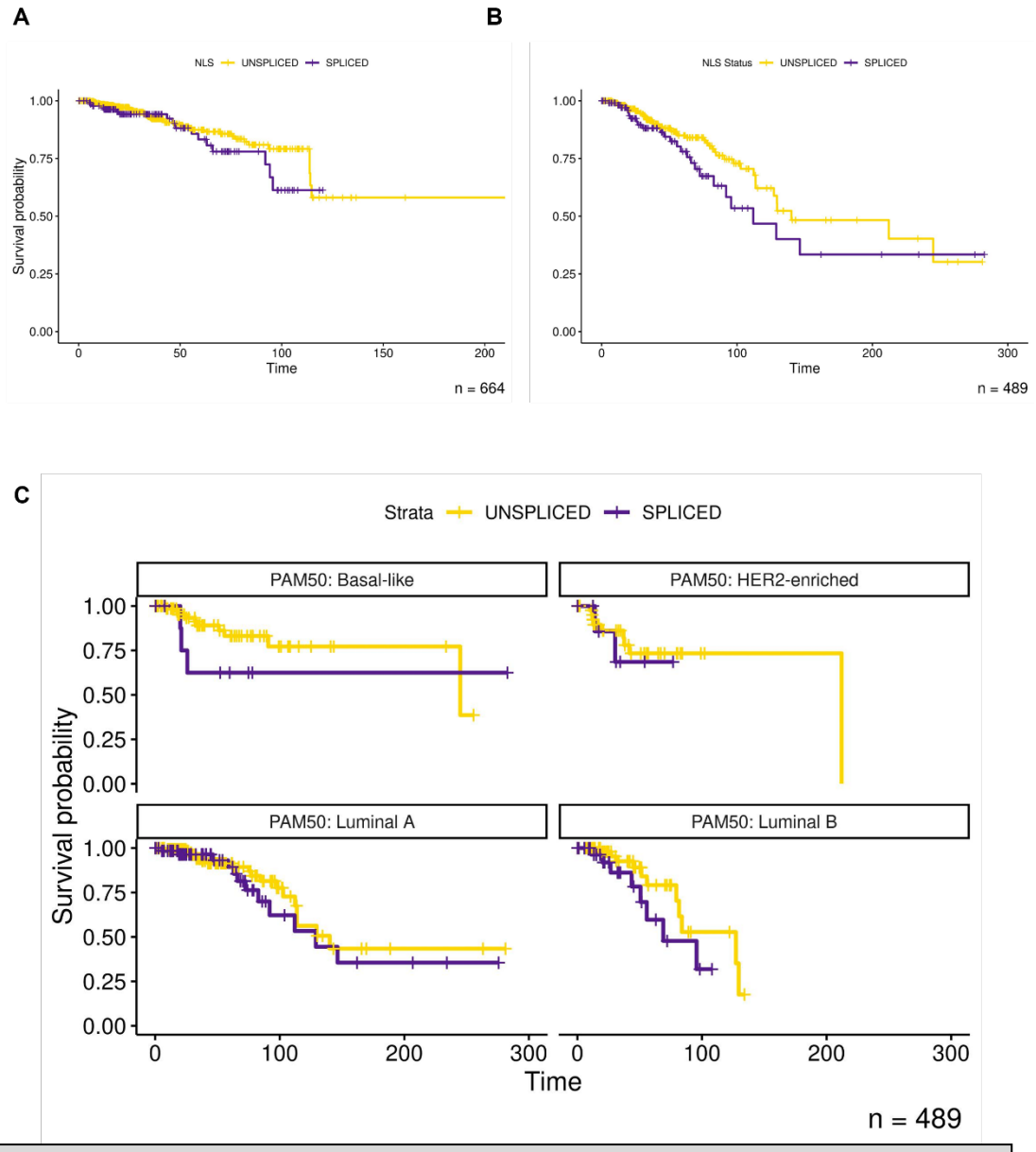

Figure 2: *NF1* NLS status correlates with diminished survival in breast cancer. A) Kaplan-Meier curve of TCGA breast cancer patients who had clinical data for all categories in Table 1 divided by expression of NF1 ΔNLS transcripts (SPICED) or no expression of ΔNLS transcripts (UNSPLICED). B) Kaplan-Meier curve of TCGA breast cancer patients with tumors that were classified into a PAM50 subtype.

and Luminal B) we saw similar trends reoccur with decreased overall survival occurring around five years after diagnosis, further supporting that ER+ ΔNLS expressing breast tumors might be correlated with endocrine resistance (Figure 2C).

	coef	exp(coef)	se(coef)	z	Pr(>|z|)
Diagnosis.Age	0.0385604	1.0393135	0.0099859	3.8614811	**0.0001127**
Mutation.Count	-0.0003078	0.9996923	0.0018872	-0.1630932	0.8704451
NLS.StatusSPLICED	0.5294098	1.6979299	0.2629651	2.0132325	**0.0440902**
NF1.StatusHETLOSS	-0.1124109	0.8936770	0.2720405	-0.4132137	0.6794501
PAM50_mRNA_nature2012HER2-enriched	0.9192579	2.5074290	0.4289063	2.1432605	**0.0320922**
PAM50_mRNA_nature2012Luminal A	-0.4800499	0.6187525	0.3743183	-1.2824643	0.1996798
PAM50_mRNA_nature2012Luminal B	0.2796885	1.3227177	0.3875582	0.7216683	0.4704985

Table 2: Cox proportional-hazard model shows *NF1* NLS status impacts overall breast cancer survival. Cox-proportional hazard model including PAM50 subtypes of breast cancers from TCGA dataset and the impact of NF1 NLS status on overall survival.

ER negative breast cancers expressing NF1 ΔNLS transcript have altered expression in splicing pathways

We sought to determine if breast tumors expressing ΔNLS transcripts were associated with a specific PAM50 subtype. We ran a chi squared test using *NF1* mutation status and *NF1* NLS status. While *NF1* indels were enriched in basal, HER2-enriched, and Luminal A subtypes, ΔNLS expression did not show any association to a specific subtype (Figure 3A, 3B, and Supplemental 2A) suggesting ΔNLS tumors may harbor unique characteristics making up its own subtype. To further investigate these results, we performed gene ontology analysis of differentially expressed genes between ER positive and ER negative spliced and unspliced tumors (Figure 3C and 3D). Knowing that ER positive spliced tumors may be prone to endocrine resistance and may switch to more of a TNBC subtype, we looked further into altered pathways in ER negative spliced tumors compared to ER negative unspliced. From our gene ontology analysis, we noticed that some of the most significantly downregulated genes are involved in the RNA splicing and the spliceosome complex.

Summary

These exciting results reveal that NF1 alternative isoforms correlate with poor prognosis in breast cancer. Importantly, our work has identified an NF1 transcript variant with exon 52 spliced out (ΔNLS) that may lead to altered nuclear localization of neurofibromin. This *NF1* ΔNLS transcript variant is highly expressed relative to other *NF1* transcript variants and correlates with poor survival in patients. We are finalizing our analysis of NF1 transcript variants in a breast cancer dataset that includes samples from metastatic and endocrine resistant breast cancers. In addition, we are performing imaging studies to understand how the *NF1* ΔNLS transcript variant alters subcellular localization and signaling of neurofibromin. We expect these studies to be complete in the next year and are preparing the data for publication.

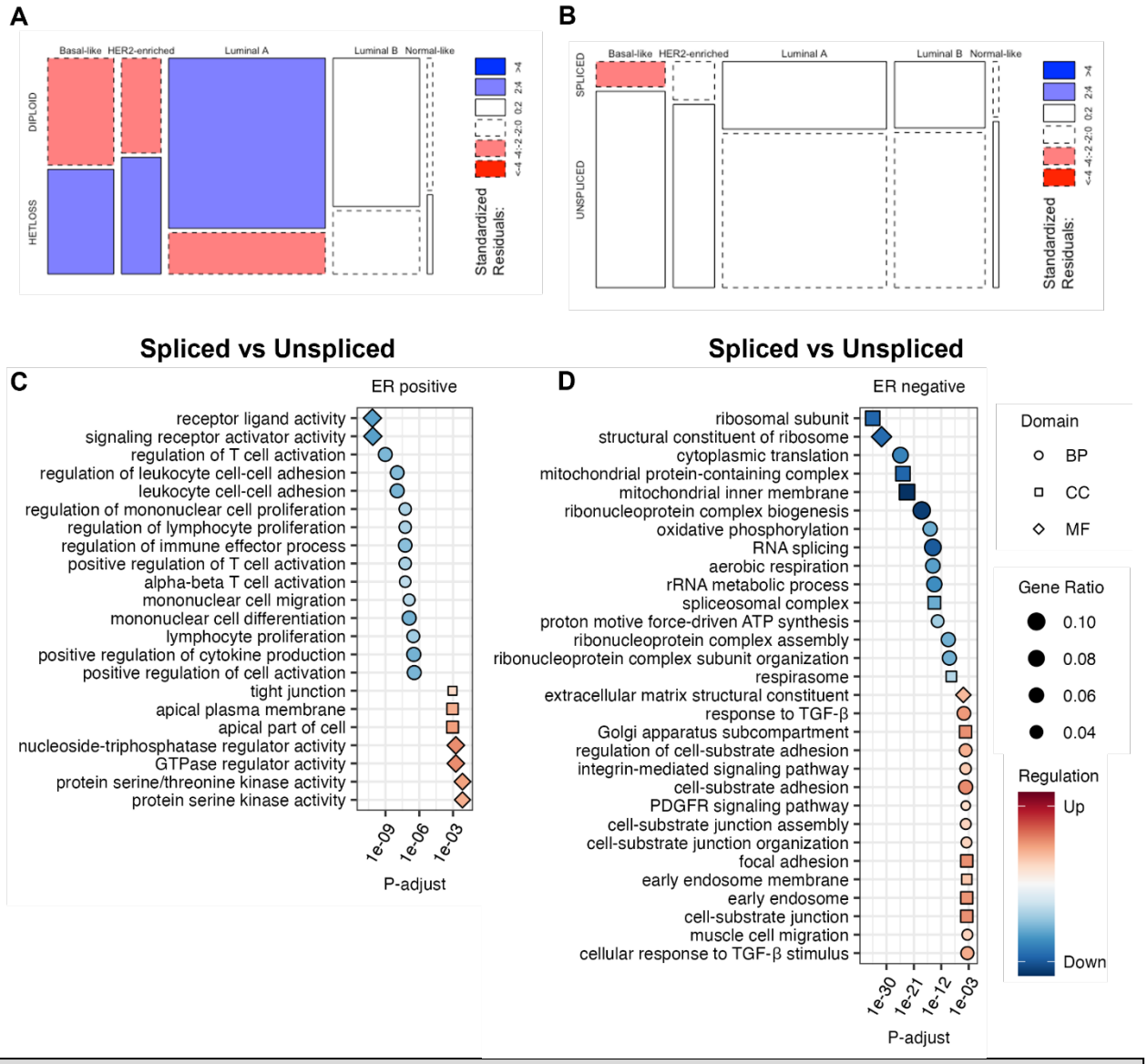

Figure 3: *NF1* NLS status is not enriched for in any breast cancer subtype.
A- B) Mosaic models of *NF1* status (A) and *NF1* NLS (B) status among breast cancer subtypes. C- D) Gene ontology dotplots of differential gene expression in ER-positive (C) and ER-negative (D) breast cancers comparing *NF1* ΔNLS expressing tumors (spliced) to non-expressing *NF1* ΔNLS tumors (unspliced).

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

How were the results disseminated to communities of interest?

We have an annual meeting with the NF-Michigan to update them on our research progress on NF1-related research. This will be held this year in March 2023. In addition, we are prearping a manuscript of this work for publication.

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

For Specific Aim 3, we were unable to perform analysis on METABRIC dataset due to the data originating from microarray analysis (compared to RNAseq data which is needed for isoform analysis). We recently received access to the AURORA dataset that includes metastatic breast cancer datasets and are completing an isoform analysis using this dataset.

In addition, we are expanding our work in Specific Aim 3 (Subtask 6: Validate NF1 isoform expression in tissues) to examine subcellular localization of NF1 isoforms in breast cancer cell lines and tissues. For this work, we have developed an NF1 ΔNLS transcript expressing cell line.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
The results of these studies will define the impact of alternative RNA splicing on *NF1* function, breast cancer progression, and therapeutic resistance. This will fill a huge gap in our understanding of *NF1* expression in normal and malignant tissues. Moreover, the results of this study may reveal RNA isoform signatures of *NF1* deficiency that could serve as diagnostic or prognostic markers for both NF1-related breast cancer and sporadic breast cancer patients.

What was the impact on other disciplines?
We expect these results to advance our understanding of the role of NF1 alternative splicing in other NF-related cancers.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
We have an annual meeting with the NF-Michigan to update them on our research progress on NF1-related research. This was held in March 2023 and we will continue to meet with this group annually. These interactions are mutually beneficial. These meetings communicate research progress in NF research and helps us understand the challenges that individuals with NF face.

5. Changes/Problems

Changes in approach and reasons for change
One of the subtasks in Aim 3 was to analyze *NF1* isoform expression in several breast cancer datasets. We were able to complete an extensive analysis of the TCGA breast cancer dataset; however, the proposed METABRIC dataset was constructed using Illumina HT 12 microarrays and not RNA-seq platforms which are required for isoform analysis. When we originally wrote this grant, the cBioportal METABRIC Datasets page inaccurately stated that the METABRIC study was composed of 1904 samples with RNA-seq and 1904 samples with mRNA microarray. Due to this fact, we have focused our bioinformatic analysis on the TCGA dataset and included *NF1*-deficient MCF7 cell lines that we have developed in the lab using CRISPR-CAS genome editing. We are also planning to analyze smaller breast cancer datasets that include metastatic samples.

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

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

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

Significant changes in use or care of human subjects *Nothing to report*

Significant changes in use or care of vertebrate animals. *Nothing to report*

Significant changes in use of biohazards and/or select agents *Nothing to report*

6. Products

Journal publications. *Nothing to report*

Books or other non-periodical, one-time publications. *Nothing to report*

Other publications, conference papers, and presentations. Poster presentation at Gordon Research Conference: Post-Transcriptional Gene Regulation, July 10 - 15, 2022; Poster presentation at Childrens Tumor Foundation 2023 NF Conference, Jun 24-27, 2023

7. Participants & Other Collaborating Organizations

Name:	Matthew Steensma
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	0.48 calendar months or 10% effort (of 40% appointment)
Contribution to Project:	Guiding the experimental design for the entire project and current overseeing the completion of experiments

Name:	Carrie Graveel
Project Role:	Senior Research Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2 calendar months or 10% effort
Contribution to Project:	Designing, performing, and analyzing the experiments; contributing to the development of research strategies, and preparing the results for presentation and publication.

Name:	Elizabeth Tovar
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.4 calendar months or 20% effort
Contribution to Project:	Designing, performing, and analyzing the experiments; contributing to the development of research strategies, and preparing the results for presentation and publication.

Name:	Patrick Dischinger
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6.0 calendar months or 50% effort
Contribution to Project:	Designing, performing, and analyzing the experiments, and bioinformatic analyses.

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 *Nothing to report*

9. Appendices *Nothing to report*