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14. ABSTRACT

Neurofibromatosis type I (NF) patients have a significantly increased risk of dying from breast cancer. Our studies have revealed previously unknown interactions between NF1, RAS deregulation, and estrogen receptor (ER) signaling. *NF1* is the key negative regulatory gene of the RAS pathway and *NF1* mutation or deletion results in aberrant RAS pathway activation. AP-1 is a heterodimeric transcription factor activated by RAS-ERK signaling that plays a pivotal role in differentiation and cell identity through enhancer binding and chromatin remodeling. In breast cancers, AP-1 can promote endocrine resistance by altering the ER cistrome and transcriptome. Together, these results strongly suggest that *NF1* loss of function promotes tumor initiation and endocrine resistance through deregulated AP and ER signaling and altered mammary progenitor differentiation. We hypothesize that NF1 deficiency promotes breast cancer and endocrine resistance in NF1-related and sporadic breast cancer by altered AP-1 and ER signaling and chromatin remodeling. This proposal will integrate novel *NF1* models and innovative technologies to resolve the role of *NF1* function in mammary development, breast cancer initiation, and endocrine-resistance. Moreover, this study may reveal RNA or chromatin signatures that could serve as diagnostic or prognostic markers for NF1-related breast cancers.

15. SUBJECT TERMS
Neurofibromatosis Type I, breast cancer, estrogen receptor, endocrine resistance

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1. Introduction

Neurofibromatosis type I (NF) patients have a significantly increased risk of dying from breast cancer. Women less than 40 years old with NF have a 10- fold increased risk of breast cancer and their lifetime risk is 2-fold higher than the general population. Importantly, NF-related breast cancers are associated with adverse prognostic factors and decreased overall survival compared to sporadic breast cancer. Our laboratory has demonstrated a critical role for *NF1* in breast cancer initiation and endocrine-resistance. We developed a novel CRISPR rat model of *Nf1* deficiency that develops aggressive mammary adenocarcinomas. These results and our human breast cancer network analysis, revealed previously unknown interactions between *NF1*, RAS deregulation, and estrogen receptor (ER) signaling. In preliminary studies, we have also observed altered mammary development and progenitor differentiation in *Nf1*-deficient mammary glands. These differentiation defects suggest that *Nf1* deficiency may alter mammary differentiation and tumor initiation.

NF1 is the key negative regulatory gene of the RAS pathway and *NF1* mutation or deletion results in aberrant RAS pathway activation. AP-1 is a heterodimeric transcription factor activated by RAS-ERK signaling that plays a pivotal role in differentiation and cell identity through enhancer binding and chromatin remodeling. In breast cancers, AP-1 can promote endocrine resistance by altering the ER cistrome and transcriptome. Together, these results strongly suggest that *NF1* loss of function promotes tumor initiation and endocrine resistance through deregulated AP and ER signaling and altered mammary progenitor differentiation. We *hypothesize* that *NF1* deficiency promotes breast cancer and endocrine resistance in *NF1*-related and sporadic breast cancer by altered AP-1 and ER signaling and chromatin remodeling.

Our proposed studies will address several areas of emphasis including 1) identification of novel disease and treatment response markers using genetics, genomics, and epigenetics; 2) target identification and drug discovery; and 3) preclinical efficacy studies for NF patients. This proposal will integrate novel *NF1* models and innovative technologies to resolve the role of *NF1* function in mammary development, breast cancer initiation, and endocrine-resistance. Moreover, this study may reveal RNA or chromatin signatures that could serve as diagnostic or prognostic markers for *NF1*-related breast cancers.

2. Keywords

Neurofibromatosis Type 1, NF1, breast cancer, endocrine resistance, estrogen receptor

3. Accomplishments

See following pages 5-11

What were the major goals of the project?

Tasks and Milestones Achieved	Timeline (Months)	Progress
Specific Aim 1: To determine the effect of *NF1* deficiency on mammary gland development and breast cancer initiation		
Major Task 1: Establish impact of *Nf1* deficiency on mammary gland differentiation and ER		
Subtask Local IRB/IACUC Approval and DoD HRPO/ACURO Approval	1-4	Completed
Subtask 1: Measure ductal branching, alveolar expansion, and TEB development in $Nf1^{WT}$, $Nf1^{IF}$, and $Nf1^{PS}$ glands.	4-12	Completed
Subtask 2: Immunostaining and confocal imaging of luminal (CK18, CK19, MUC1) and basal (CK14, VIM, SMA) lineage markers.	1-12	Completed
Subtask 3: Co-immunostaining and confocal imaging glands with NF1-ER, NF1-pER, and NF1-pERK antibodies in 21 and 33 day old mammary glands.	1-12	In progress
Major Task 2: Determine role of Nf1 in lineage commitment and progenitor differentiation		
Subtask 1: Determine epithelial cell-state proportions by fluorescence-activated cell sorting (FACS) in $Nf1^{WT}$, $Nf1^{IF}$, and $Nf1^{PS}$ glands.	1-12	Completed
Subtask 2: Targeted qRT-PCR on the sorted populations for luminal, basal, and progenitor/stem marker expression.	1-12	Completed
Subtask 3: Immunostaining of lineage markers and ER target genes in sorted cell populations.	1-12	Completed
Major Task 3: Define role of NF1 on differentiation and ER signaling in NF1-deficient MCF10A		
Subtask 1: Perform 3D cultures of *NF1*-deficient MCF10A cells and measure luminal and basal marker expression with quantitative RT-PCR and IF imaging.	6-18	Completed
Subtask 2: In *NF1*-deficient MCF10A cells, determine correlation of *NF1* functionality with viability, motility, and invasion assays.	4-18	In progress
Subtask 3: Measure ER and AP-1 target gene expression using quantitative RT-PCR.	20-30	In progress
Specific Aim 2: To examine the impact of *NF1* deficiency on chromatin dynamics and endocrine resistance.		
Major Task 1: ChIP-seq and ATAC-seq in *Nf1*-deficient breast cells.		
Subtask 1: Perform directed ChIP-seq using H3K4me4 and H3K27ac antibodies to identify active and poised enhancers.	4-15	In progress
Subtask 2: Perform directed ChIP-seq using ER and AP-1 antibodies to identify active and poised enhancers.	6-18	In progress
Subtask 3: Validate results with direct ChIP on ER and AP-1 target	12-24	In progress

genes		
Subtask 4: Perform ATAC-seq on *Nf1*-deficient MECs and MCF10A cells.	9-24	Pending
Major Task 2: ChIP-seq and ATAC-seq in SERD-resistant Nf1-deficient tumors.		
Subtask 1: Perform directed ChIP-seq using H3K4me4 and H3K27ac antibodies to identify active and poised enhancers in SERD-sensitive and -resistant tumors	18-32	Pending
Subtask 2: Perform directed ChIP-seq using ER and AP-1 antibodies to identify active and poised enhancers.	18-32	Pending
Subtask 3: Validate results with direct ChIP on ER and AP-1 target genes.	18-32	Pending
Subtask 4: Perform ATAC-seq on SERD-sensitive and -resistant tumors.	18-32	Pending
Major Task 3: RNA-seq in Nf1-deficient breast cells and SERD-resistant Nf1-deficient tumors.		
Subtask 1: Perform RNA-Seq in *Nf1*-deficient MECs,MCF10A cells, and tumors.	12-24	Completed
Subtask 2: Gene set enrichment analysis (GSEA) and bioinformatic analysis to correlate ER signatures with *Nf1* functionality.	15-36	Completed
Specific Aim 3: To determine the efficacy of SERD and/or MEK inhibition in preventing *Nf1*-deficient breast cancer and *Nf1*-mediated chromatin remodeling.		
Major Task 1: Prevention of differentiation defects in Nf1-deficient rats using SERD and MEK inhibition.		
Subtask 1: Measure tumor prevention efficacy of SERD and /MEK inhibitor treatments in our *Nf1*-deficient rat tumors.	18-24	Pending
Subtask 2: Harvest mammary glands and assess pathological features.	24-26	Pending
Major Task 2: Effect of ER and MEK inhibition on *Nf1*-deficient mammary differentiation and chromatin remodeling.		
Subtask 1: FACS sort MECs from each treatment group and evaluate luminal and basal cell populations by CD29/CD24 expression.	18-36	Pending
Subtask 2: ATAC-seq on sorted populations to measure chromatin accessibility in each treatment group.	18-36	Pending
Subtask 3: Targeted quantitative RT-PCR will be performed to assess expression of ER and AP-1 target genes.	18-36	Pending

What was accomplished under these goals?

Specific Aim 1: To determine the effect of *NF1* deficiency on mammary gland development and breast cancer initiation. We *hypothesize that NF1* deficiency alters mammary progenitor differentiation, luminal/basal lineage commitment, and promotes tumor initiating populations. To determine the mechanisms by which *NF1* deficiency promotes tumor initiation in the breast, it is essential to understand the mechanistic role of *NF1* in normal mammary development.

Major Task 1: Establish impact of *Nf1* deficiency on mammary gland differentiation and ER

To understand how *Nf1* deficiency promotes tumor initiation in the breast, we explored the impact of *Nf1* indels and neurofibromin function on mammary morphogenesis. To start, we examined several timepoints of postnatal mammary development in our *Nf1*-deficient rat models (Figure 1A). Mammary gland whole mounts from the 4th mammary fat pad were analyzed at postnatal day 21 which represents the start of puberty. At this timepoint, the development and expansion of both the 4th and 5th ductal trees into the mammary fat pad can be observed (Figure 1B). Examination of the ductal outgrowth patterns in 21-day old females demonstrated a substantial increase in ductal expansion in all the *Nf1*-deficient lines compared to $Nf1^{WT/+}$ mammary pads (Figures 1B-1C). At postnatal day (PND) 21, the $Nf1^{WT/+}$ ductal outgrowths had elongated to approximately 2/3 of the distance from the nipple to the lymph node (LN). In contrast, both $Nf1^{IF/+}$ and $Nf1^{PS\text{-}20/+}$ females demonstrated accelerated ductal expansion that reached or surpassed the 4th mammary gland LN (Figure 1B-1C). Even though the $Nf1^{IF;PS\text{-}21/+}$ ductal expansion did not extend longitudinally to the extent of that in $Nf1^{IF/+}$ or $Nf1^{PS\text{-}20/+}$ ductal outgrowths, the $Nf1^{IF;PS\text{-}21/+}$ pads showed expansive ductal filling throughout the fat pad compared to the $Nf1^{WT/+}$ and other *Nf1*-deficient lines (Figure 1B-C). Automated quantitation revealed a significant increase in the percent of epithelial cells present in the 4th mammary fat pads in all the *Nf1*-deficient lines compared to $Nf1^{WT/+}$ glands ($p < 0.0001$; Figure 1D). These results imply that *NF1* plays a role in regulating mammary gland development.

To investigate the impact of *Nf1* deficiency on ductal elongation, we evaluated both the structural patterns of ductal branching and terminal end buds (TEBs). TEBs are bulbous formations at the tips of developing ducts that are completely unique to the peripubertal mammary gland and believed to contain mammary stem cells (MaSCs). The MaSCs within the TEB are responsible for the production of the mature cell types that result in elongation of the subtending duct (1). Carmine staining on wholemounts of the mammary glands demonstrated striking differences in both the ductal and TEB structures in the *Nf1*-deficient lines (Figure 1E). At 21 days, the primary ductal branches had no signs of lateral ductal budding in the $Nf1^{WT/+}$ mammary pad, whereas there was profuse budding on subtending primary ducts in the *Nf1*-deficient lines (Figure 1E, middle panel). The TEBs in the $Nf1^{IF/+}$ line were slightly larger than those in the $Nf1^{WT/+}$ mammary gland, and the TEBs in the $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ lines were a mix of large TEBs and small, grape-like clusters of aggregated buds pointed in multiple directions, indicating disorganized directional formation coupled with aberrant cell growth (Figure 1E, right panel). Blinded quantification of the total number of buds, both TEBs and lateral, revealed an increased number of buds in the *Nf1*-deficient lines compared to $Nf1^{WT/+}$ (Figure 1F). To get a better understanding of the timeline in which *Nf1* influences mammary development we also assessed glands at 8 and 33 PND (data not shown). Accelerated ductal outgrowth and aberrant alveolar budding is present at 8 PND in *Nf1*-deficient mammary glands. At 33 PND, the *Nf1*-deficient mammary glands have pervaded throughout the fat pad more extensively than $Nf1^{WT/+}$ glands. These findings demonstrate that *Nf1* deficiency results in accelerated mammary development that is mitigated by the level of *Nf1* functional loss, such that $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ mammary glands have more extensive aberrant ductal growth compared to $Nf1^{IF/+}$ mammary glands at these early stages of development.

To gain further insight into the impact of *Nf1* function on pubertal mammary development, we focused on the cells comprising the 21 day TEB. The inner mass of a TEB is made of body cells, which are presumed to be luminal ductal and alveolar progenitors The single-cell layer lining the TEB are composed of cap cells, which give rise to myoepithelial cells. Cap cells have been shown to have regenerative stem cell capabilities in transplantation assays, but other studies have contradicted these results and whether cap cells are MaSCs or restricted progenitors is still debated (2). A combination of epithelial and stromal signals are essential for maintaining TEB unidirectional and non-overlapping growth (3-5). High magnification images showed that $Nf1^{IF/+}$ TEBs are nearly indistinguishable from $Nf1^{WT/+}$ TEBs, except for the presence of larger TEBs within close proximity (Figure 2A). The TEBs from $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ lines were considerably distorted compared to $Nf1^{WT/+}$ glands. Notably, TEBs from $Nf1^{PS\text{-}20/+}$ glands had a thickened body cell layer while $Nf1^{IF;PS\text{-}21/+}$ TEBs were an amalgamation of normal and completely disorganized buds that resembled the terminal ductal-lobular units present in adult human glands (6). Note that similar tubuloalveoar formations are not observed in rats until after

post-natal day 46, which is beyond the 21 day timepoint shown in Figure 2 (7). These results suggest that *Nf1* deficiency perturbs TEB formation and consequently ductal outgrowth in mammary development.

To elucidate how *Nf1* deficiency alters TEB structure and cell proliferation, we immunostained mammary gland sections from 21 PND females with distinct, well-defined lineage markers: smooth muscle actin (SMA, green) for basal/myoepithelial/cap cells and E-cadherin (red) for luminal epithelial/body cells, combined with Ki67 to mark proliferating cells (blue) (Figure 2B-C) (2). Compared to $Nf1^{WT/+}$, the overall structure of $Nf1^{IF/+}$ TEBs was comparable with similar expression patterns of E-cadherin in the TEB body and SMA at the TEB periphery (Figure 2B). In contrast, $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ TEBs had diffuse and disorganized E-cadherin and SMA expression throughout the TEB. As shown in the magnified images of the $Nf1^{WT/+}$ TEB in Figure 2C, body cells are enveloped by a contiguous single-cell layer of cap cells. The rare cap cells that are found in the body cell layer are hypothesized to be left behind after TEB outgrowth (8) and undergo cell death (2,9). In $Nf1^{PS\text{-}20/+}$ TEBs, we observed decreased and diffuse SMA staining at the periphery and the presence of SMA+/Ecad+ cells within the body cell layer. A similar, but more pronounced phenotype was observed in $Nf1^{IF;PS\text{-}21/+}$ glands, where E-cadherin expression was diminished throughout the TEB body and numerous SMA/Ecad+ cells were observed (Figure 2B-C). Evaluation of cap cell proliferation in the *Nf1*-deficient TEBs revealed a marked increase in Ki67 staining intensity in cap cell nuclei in all the *Nf1*-deficient lines compared to $Nf1^{WT/+}$, yet the strongest Ki67 staining was present in $Nf1^{IF/+}$ and $Nf1^{IF;PS\text{-}21/+}$ TEBs (Figure 2B). We also detected Ki67 staining in the cap cells within the TEB body with weak E-cadherin staining (Figure 2C). The increased proliferation was verified by immunohistochemical staining of Ki67 in cross-sections of the developing mammary glands. Together the presence of SMA+/Ecad+ cells, decreased E-cadherin expression, and altered cap cell localization suggests that *Nf1* deficiency impacts epithelial differentiation within the developing mammary gland.

Major Task 2: Determine role of *Nf1* in lineage commitment and progenitor differentiation

<u>*Nf1* deficiency induces a shift in luminal to basal epithelial populations</u>

Methods to characterize the epithelial hierarchy have been well established in the human and mouse mammary glands (10). To delineate whether *Nf1* deficiency alters the mammary epithelial hierarchy, we modified and optimized these methods for rat tissues (11). We isolated mammary epithelial cells (MECs) by enzymatic digestion of the 4th mammary fat pads from 22 and 43 day old female rats, and then subjected the samples to FACs (fluorescence-activated cell sorting). Briefly, endothelial and hematopoietic cells were depleted from freshly digested samples using the lineage markers CD31 and CD45 respectively. The resulting lineage negative (Lin^-) population was gated into three distinct subpopulations using rat-specific antibodies against the cell adhesion molecule CD24 (cluster of differentiation 24) and CD29 (integrin beta-1), confirming previous gating results (7) (Figure 3A). Three distinct populations were identified that represented luminal ($CD24^{hi}/CD29^{mid}$), basal ($CD24^{mid}/CD29^{hi}$), and stromal ($CD24^{low}/CD29^{hi}$) cells. We discovered there was a significant increase in luminal cells in the $Nf1^{IF/+}$ and $Nf1^{IF;PS\text{-}21/+}$ glands at 22 days, yet at 43 days, a significant decrease in the luminal population was detected in the $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ glands (Figure 3B). For the basal population, a significant increase in the basal cell population was observed in $Nf1^{IF;PS\text{-}21/+}$ glands at 22 days, while a positive trend was present in $Nf1^{IF/+}$ and $Nf1^{PS\text{-}20/+}$ glands (Figure 3C). By 43 days, both the $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ glands had a significant increase in basal population countered by a significant decrease in the luminal population. The results demonstrate a preferential expansion of the basal cell compartment between 22 and 43 days in *Nf1*-deficient glands. Notably, the $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ rats that have a shift in luminal-basal lineages also have the most aggressive phenotype. We observed a decrease in the basal cell population at 43 days in $Nf1^{IF/+}$ mammary glands, which may produce the delayed tumor onset observed in this line (~10 mos). Collectively, these data reveal a shift in luminal to basal lineage commitment within the *Nf1*-deficient lines that have the earliest tumor onset.

To validate these results, we assessed lineage specific protein expression in each genotype at 22 and 43 days. Following FACs, cytospun cells were immunostained with lineage markers (Figure 3D). As expected, $Nf1^{WT/+}$ luminal cells were CK18+ and basal cells were SMA+ at both 22 and 43 days; whereas $Nf1^{IF;PS\text{-}21/+}$ luminal cells had decreased CK18 and mild SMA expression at both timepoints. We also assessed ER which is expressed normally in luminal populations. In addition, we examined phosphorylated S118 ER, the ERK phosphorylation site that facilitates coactivator interactions and can be impacted by RAS dysregulation (12,13). $Nf1^{WT/+}$ luminal cells were ER+ and pER+ at 22 days, yet decreased ER+ and pER+ expression was observed at 43 days, whereas basal cells were negative for both ER and pER. In $Nf1^{IF;PS\text{-}21/+}$ glands pER(S118) was strongly expressed

in both luminal and basal cells. Together the shift in luminal to basal populations, the co-expression of luminal and basal lineage markers within *Nf1*-deficient mammary epithelium demonstrates that *Nf1* functionality impacts mammary lineage commitment.

Nf1-deficency promotes lineage plasticity within mammary glands

To examine the impact of *Nf1* deficiency on cell fate later in mammary development and throughout tumorigenesis, we immunostained $Nf1^{WT/+}$, $Nf1^{PS\text{-}20/+}$, or $Nf1^{IF;PS\text{-}21/+}$ mammary glands at several timepoints with E-cadherin and SMA (Figure 4). To identify cells that express both luminal and basal markers, we used an image calculator in FIJI. For E-cadherin and CK14 image masks, a pre-determined threshold was applied to each channel (based on multiple training sets of images taken from all genotypes) to identify areas of lineage marker overlap. In mammary glands from 22d old females, we observed few dual positive ($Ecad^+/CK14^+$) cells within the $Nf1^{PS\text{-}20/+}$ ducts; however, a substantial number of $Ecad^+/CK14^+$ cells were present in the $Nf1^{IF;PS\text{-}21/+}$ ducts (Figure 4A). Next we evaluated mammary glands at >50 days, in which the *Nf1*-deficient glands had hyperplastic changes but had not developed tumors (Figure 4B). At >50d, the $Nf1^{PS\text{-}20/+}$ and $Nf1^{IF;PS\text{-}21/+}$ glands exhibited varying levels of $Ecad^+/CK14^+$ cells, yet these dual-positive cells were not present in any $Nf1^{WT/+}$ glands (Figure 4B). As opposed to the patterns observed at 22d, the $Ecad^+/CK14^+$ cells in the >50d glands were consistently found at the periphery of the ductal structures consistent with the basal cell layer.

Next we examined whether these $Ecad^+/CK14^+$ cells persisted throughout tumor development. The expression patterns that we observed in the hyperplastic glands led us to question whether $Ecad^+/CK14^+$ cells are present within different tumor regions. To evaluate localization of the luminal/basal populations, we imaged the middle, edge, and invasive edge of each tumor. In an $Nf1^{PS\text{-}20/+}$ tumor, we observed few $Ecad^+/CK14^+$ cells in the middle of the tumor and edge of the tumor, but as in the hyperplastic glands, the $Ecad^+/CK14^+$ cells were located in either the basal layer or at the invasive edge of the tumor (Figure 4C, left panel). Strikingly, the strongest expression overlap was observed in tumor cells that had infiltrated into the stromal. This was also observed in an $Nf1^{IF;PS\text{-}21/+}$ tumor where $Ecad^+/CK14^+$ cells were identified in the stroma infiltrates, yet very weak signal was detected $Ecad^+/CK14^+$ cells within the tumor middle and invasive edge (Figure 4C, right panel). Together these results demonstrate that $Ecad^+/CK14^+$ cells are present in *Nf1*-deficient mammary glands at the earliest stages of development, yet they are a minute subpopulation. In the earliest stages of tumor development, these luminal/basal populations are located at the basal layer, yet later are only observed at the invasive edge and infiltrating cells. These findings imply that *Nf1*-deficient tumors contain a subpopulation of cells with lineage plasticity between the luminal-basal epithelial states.

Major Task 3: Define role of *NF1* on differentiation and ER signaling in *NF1*-deficient MCF10A

To evaluate the effect of *NF1* loss of function in human breast cells, we used the immortalized, nontumorigenic human breast epithelial cell line MCF10A. CRISPR guides targeting exon 21 within the GRD domain were constructed and used either as single or dual-guide RNAs with Cas9 nuclease. We established three cell lines (i.e., g1, g2, g1/2) and *NF1* loss was validated by PCR and Western blot analysis. MCF10A cells were grown in 3D culture conditions and, as expected, the empty vector (EV) MCF10A line developed small spherical 3D colonies (Figure 5A). In contrast, MCF10A lines with *NF1* mutations had distinct phenotypes, with the NF1-g2 and NF1-g1/g2 lines developing larger, grape-like clusters. Interestingly, the NF1-g1 line formed few, smaller 3D colonies with the majority surviving as single cells. Immunostaining showed changes in expression of E-cadherin and CK14 in the NF1-g2 and NF1-g1/g2 cells. NF1-g1 colonies weakly expressed E-cadherin and strongly expressed CK14 suggesting these cells have become more basal-like. We performed RNAseq and utilized principal component analysis (PCA) to visualize the variability across the MCF10A-$NF1^{mut}$ cell lines (Figure 5B). PCA displayed the clonal diversity in the parental MCF10A lines and the global shift in gene expression in the *NF1*-mutant lines compared to the EV. Next we examined the impact of *NF1* deficiency on the MCF10A transcriptomes within previously defined luminal and basal classifier gene signatures (14,15) and observed that *NF1* deficiency induced consistent expression changes in both luminal and basal gene sets (Figure 5C). MCF10A-$NF1^{mut}$ lines upregulated several genes in the luminal signature known to promote cancer progression or correlate with poor prognosis (EFDH1, MLPH, ALDH6A1) (16-18). Notably, several genes were deregulated that are involved in ERα signaling and cofactor regulation (PBX1, RhoB, FOXA1, CA12). In the basal gene signature, MCF10A-$NF1^{mut}$ lines showed downregulation of genes involved in cell adhesion (KRT6B, KRT17, DSC3, JAG). Two of the most upregulated genes in the basal signature are metabolic genes that protect against oxidative stress (CBS) (19) and are involved in the pyrimidine salvage

pathway (AOBECD3B) (20,21). We observed similar gene expression changes in the MCF10A-*NF1*^{mut} lines except in one subclone in the NF1-g2 line (Figure 5B-C). Because of the Ecad^{+}/CK14^{+} cells we observed in *Nf1*-deficient tumors (Figure 4C), we evaluated the expression changes in several genes that are essential for EMT and saw that the clones that often did not form 3D mammospheres (NF1-g1 and NF1-g2) had lost E-cadherin and Desmoplasia expression but had increased mesenchymal adhesion proteins (Cdh2, Vimentin, and Fibronectin) and the EMT transcription factors *Twist1* and *Zeb1* (Figure 5D). Using gene set enrichment analysis (GSEA), we found that several KEGG pathways were significantly enriched in the MCF10A-*NF1*^{mut} lines compared to the EV control, including Pathways in Cancer (includes RAS signaling), regulation of actin cytoskeleton, focal adhesion, and cytokine-cytokine receptor interaction) (data not shown). The enrichment of these pathways in *NF1*-deficient cells aligns with the EMT signatures (Figure 7D) and gene expression changes we observed (Figure 5C).

Specific Aim 2: To examine the impact of *NF1* deficiency on chromatin dynamics and endocrine resistance. We *hypothesize* that *NF1* deficiency results in enhancer reprogramming at AP-1 and ER transcriptional binding sites leading to altered mammary differentiation, breast cancer initiation, and endocrine resistance. To dissect the role of *NF1* in ER signaling we will assess the impact of *NF1* loss on mammary differentiation and in endocrine resistance, we will examine the chromatin landscape of ER-sensitive and ER-resistant *Nf1*-deficient tumors.

Major Task 1: ChIP-seq and ATAC-seq in Nf1-deficient breast cells

We isolated MECs from *Nf1*^{WT}, *Nf1*^{IF}, and *Nf1*^{PS} rats and *MCF7* NF1+/- cells. MECs were isolated from normal mammary glands at day 33, at which time we observe abnormal mammary development in the *Nf1*-deficient lines, yet malignant features are not present. In this grant period, we have optimized several steps throughout the extensive ChIP-seq protocol including DNA fragmentation size and immunoprecipitation. While optimizing conditions, we discovered that the ER antibody we had been using (Ab-10, Thermo MS-315-PABX, selected based on use in Carroll et al. 2006) was no longer commercially available. Therefore, we had to turn to the literature to identify and test a handful of alternate estrogen receptor antibodies to see if they could pull down ER-DNA complexes both with and without estrogen in our system. We are now starting our first large scale ChIP-seq experiments with ER and AP-1 antibodies.

Major Task 3: RNA-seq in Nf1-deficient breast cells and SERD-resistant Nf1-deficient tumors.

We have developed two models of Nf1-realted endocrine resistance. Using CRISP-Cas9 genome editing, we have created *NF1*-deficient MCF7 cell lines that have variable responses to estrogen stimulation and selective estrogen receptor degraders (SERDs) treatment. In addition, we have developed three *Nf1* tumorgraft lines that are resistant to endocrine therapy. To explore the relationship between *NF1* loss of function and the ER transcriptome, we performed RNA-seq on the following cell lines and tumors that were harvested after treatment with SERDs: 1) *MCF7* NF1+/-, 2) *Nf1*-deficient tumors, and 3) SERD-resistant *Nf1*-deficient tumors. Libraries were sequenced using 50 bp, paired end on an Illumina NovaSeq6000 sequencer for 50M reads/library. The resulting RNA-seq results are being analyzed by bioinformatic scientists in the VAI Bioinformatics Core.

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 September 2022. In addition, we expect to have at least one manuscript in submission for publication at the end of the next grant period.

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

Our plans for each Aim are discussed above with each aim.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
The recent studies demonstrating a role for NF1 in endocrine-resistance and the high incidence of breast cancer in NF1 patients highlights the fundamental need for a more comprehensive understanding of NF1 functions within the breast and other tissues. Our studies demonstrate that *Nf1* deficiency dramatically accelerates breast cancer development and altered breast tissue organization. In addition to accelerated development and altered organization, we observed a shift in luminal to basal lineage commitment within the *Nf1*-deficient tissues that correlated with early tumor onset. Together these results reveal a mechanism by which *NF1* deficiency in NF1 patients promotes breast cancer initiation and increases breast cancer risk.

What was the impact on other disciplines?
We expect these results to advance our understanding of the role of NF1 on estrogen signaling and endocrine resistance in both NF1-related and sporadic breast 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 will be held this year in September 2022. 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

While optimizing conditions for ChIP, we discovered that the estrogen receptor antibody we had been using (Ab-10, Thermo MS-315-PABX, selected based on use in Carroll et al. 2006) was no longer commercially available. Therefore, we had to turn to the literature to identify and test a handful of alternate estrogen receptor antibodies to see if they could pull down ER-DNA complexes both with and without estrogen in our system.

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. We have a manuscript in preparation for submission in October 2022.

7. Participants & Other Collaborating Organizations

Name:	Matthew Steensma
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	0.70 calendar months or 15% 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:	4.2 calendar months or 35% 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.

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

A. References
B. Figure Legends
C. Figures 1 – 5
D. Other Support

References

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Figure Legends

Figure 1: *Nf1* deficiency promotes aberrant ductal outgrowth and alveolar expansion during mammary morphogenesis. A) Schematic of wild-type and mutant neurofibromin in *Nf1*-deficient rat lines. The black bar within the GRD denotes indel location. Immunohistochemistry H&E of wild-type and *Nf1*-deficient glands at B) 5x [scale bar = 5mm] and C) 20x magnification [scale bar = 1mm]. The dashed black line indicates the tip of the ductal tree in relation to the lymph node. D) Percent of epithelial cells quantified per genotype (n=4). E) Carmine stains of whole mammary fat pads from 21-day old wild-type and *Nf1*-deficient rats showing the entire ductal tree at 20x (first column, scale bar = 1000 μm), ductal branches at 80x (second column, scale bar = 500 μm), and alveolar buds/TEBs (last column) at 80x magnification. F) Total alveolar and TEB counts quantified per genotype (WT vs. IF $p = 0.0001$, WT vs. PS-20 or IF;PS-21 $p<0.0001$; n=5).

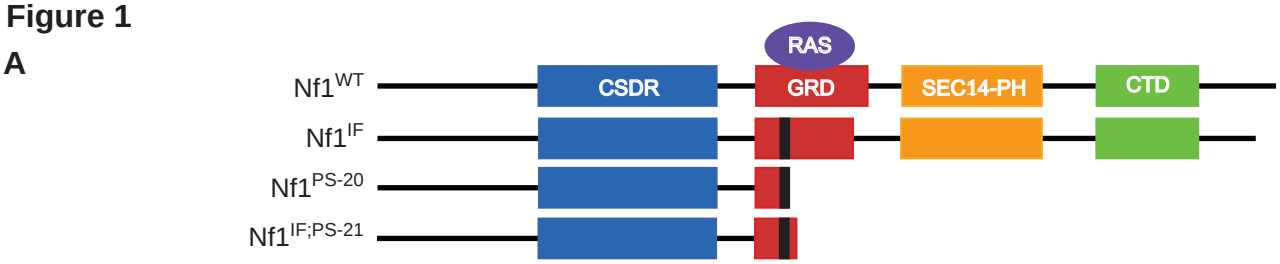
Figure 2: *Nf1* indels impact mammary morphogenesis and TEB formation. A) Immunohistochemistry H&E of TEBs from 21-day old female rats. Images were taken at 200x magnification, scale bar = 100 μm. B) Immunofluorescent confocal images of TEBs from 21-day old female rats immunostained with E-cadherin (red), SMA (green), and Ki67 (blue). Images were acquired at 600x magnification, scale bar = 21 μm. C) Insets from part B 'Merge' column. Upper row shows cells in the CAP cell layer and the bottom row shows cells in the body cell compartment.

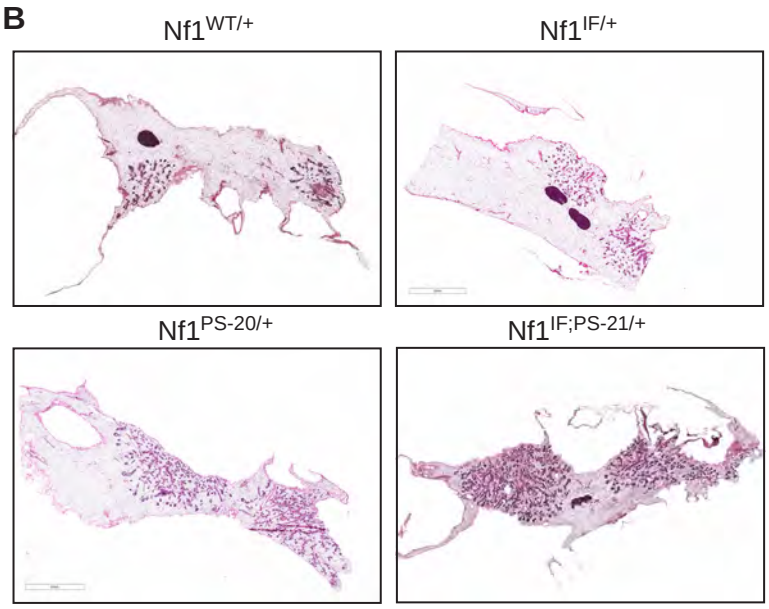
Figure 3: Mammary lineage population and marker expression changes in *Nf1* deficient rats. A) Flow cytometry analysis of digested mammary fat pads sorted on CD24 (luminal marker) and CD29 (basal marker) at 22 and 43 days in wild-type versus *Nf1*-deficient rat lines. B&C) The percent population of flow cytometry sorted B) luminal cells and C) basal cells at 22 and 43 days per genotype (n=5 animals per genotype; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). D) Immunostaining of luminal and basal cells spun onto glass slides following FACs from wild-type and *Nf1*-deficient female rats. Cells were stained for CK18, ER, pER, and SMA. Images were taken at 200x (IHC) and 600x (IF) magnification and insets are shown.

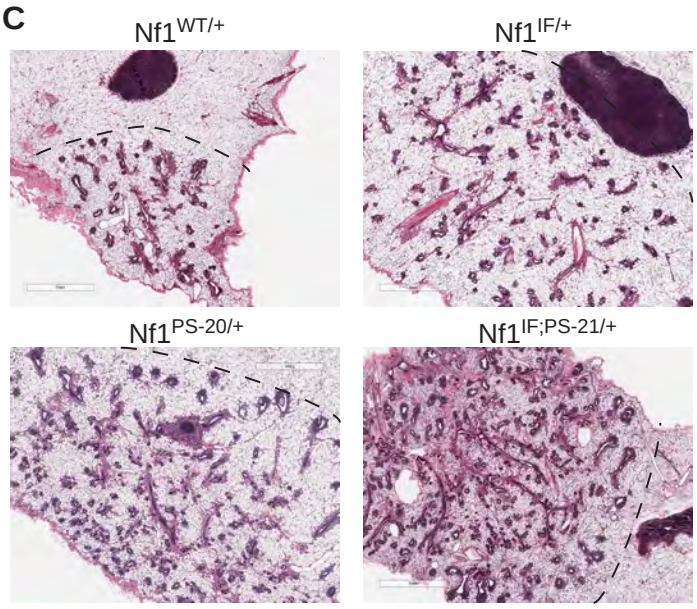
Figure 4: Dual lineage marker expression increases during development in *Nf1*-deficient mammary glands. Mammary tissues from wild-type and *Nf1*-deficient female rats were immunostained with CK14, Ecadherin, and DAPI at A) 22 days old, B) > 50 days old, and C) after mammary adenocarcinoma development. The top row in each panel represents merged CK14, E-cadherin, and DAPI staining. The bottom row in each panel represents FIJI image calculation of areas with CK14 AND E-cadherin overlay (white), indicating co-expression. Images were acquired at 600x magnification, scale bar = 21 μm.

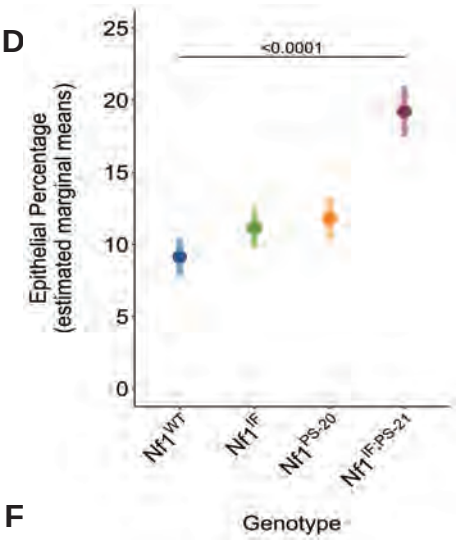
Figure 5: *NF1* Deficiency induces EMT. A) MCF10A *NF1* CRISPR mutants and control were grown in Matrigel for 10 days and then stained with CK14, E-cadherin, and DAPI. Images were taken at 600x, scale bar = 21μm. B) Principal component analysis of MCF10A *NF1* CRISPR mutants compared to empty vector and parental controls showing variation in samples from RNA sequencing analysis. C - D) Heatmaps of *NF1* CRISPR mutants (log fold change vs. the empty vector control) showing C) consistent changes in RNA expression of proteins from both luminal and basal gene signatures and D) changes in RNA expression of proteins related to EMT. E) Schematic demonstrating the impact of Nf1 loss of functionality on normal mammary development through shifting both luminal to basal differentiation states and epithelial to mesenchymal states.

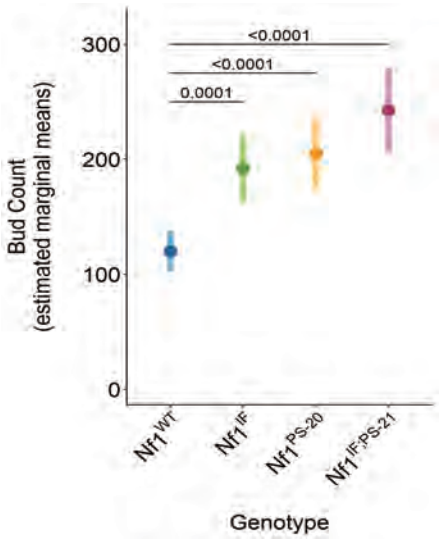
Figure 1


A
RAS
CSDR
GRD
SEC14-PH
CTD
Nf1WT
Nf1IF
Nf1PS-20
Nf1IF;PS-21


B
Nf1WT/+
Nf1IF/+
Nf1PS-20/+
Nf1IF;PS-21/+


C
Nf1WT/+
Nf1IF/+
Nf1PS-20/+
Nf1IF;PS-21/+


D
<0.0001
Epithelial Percentage
(estimated marginal means)
25
20
15
10
5
0
Nf1WT
Nf1IF
Nf1PS-20
Nf1IF;PS-21
Genotype


F
<0.0001
<0.0001
0.0001
Bud Count
(estimated marginal means)
300
200
100
0
Nf1WT
Nf1IF
Nf1PS-20
Nf1IF;PS-21
Genotype

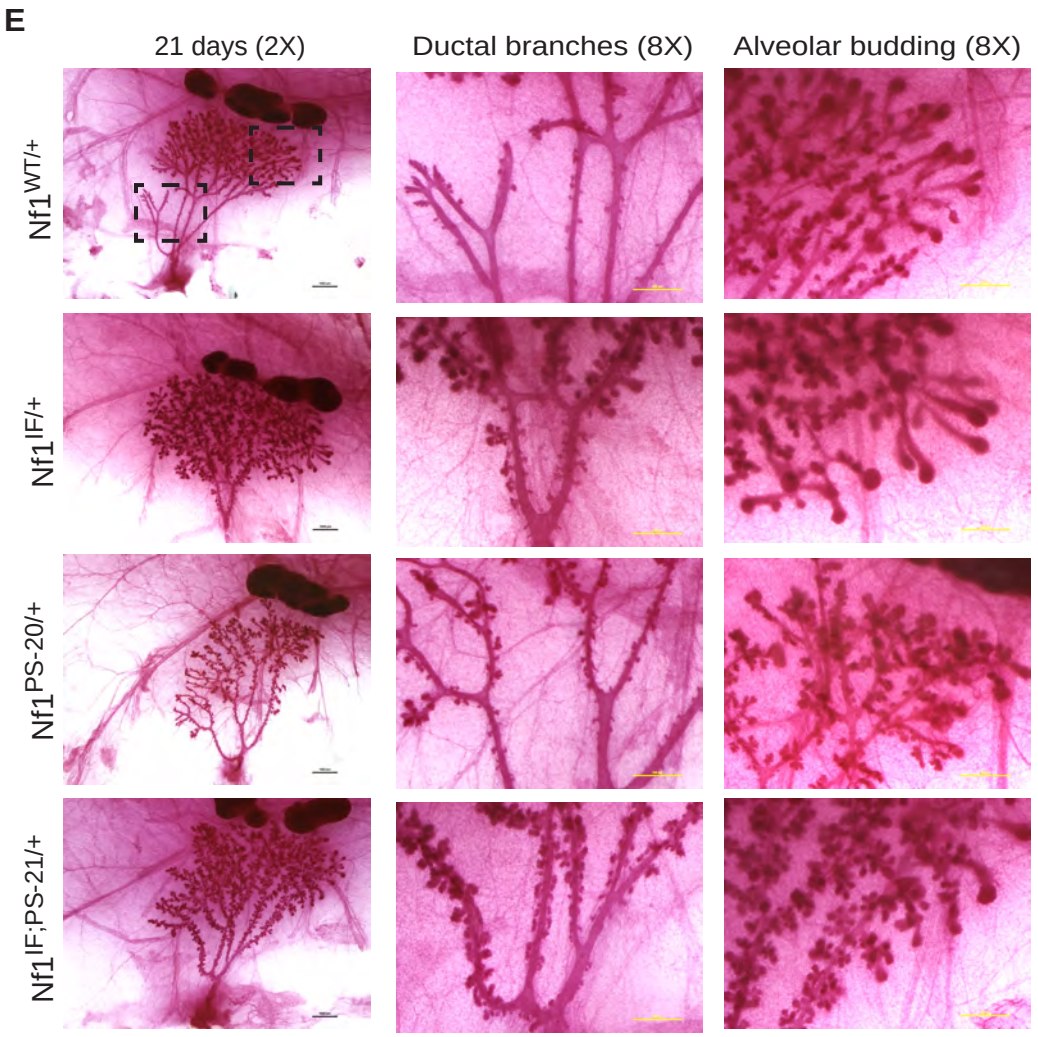

E
21 days (2X)
Ductal branches (8X)
Alveolar budding (8X)
Nf1WT/+
Nf1IF/+
Nf1PS-20/+
Nf1IF;PS-21/+

Figure 2

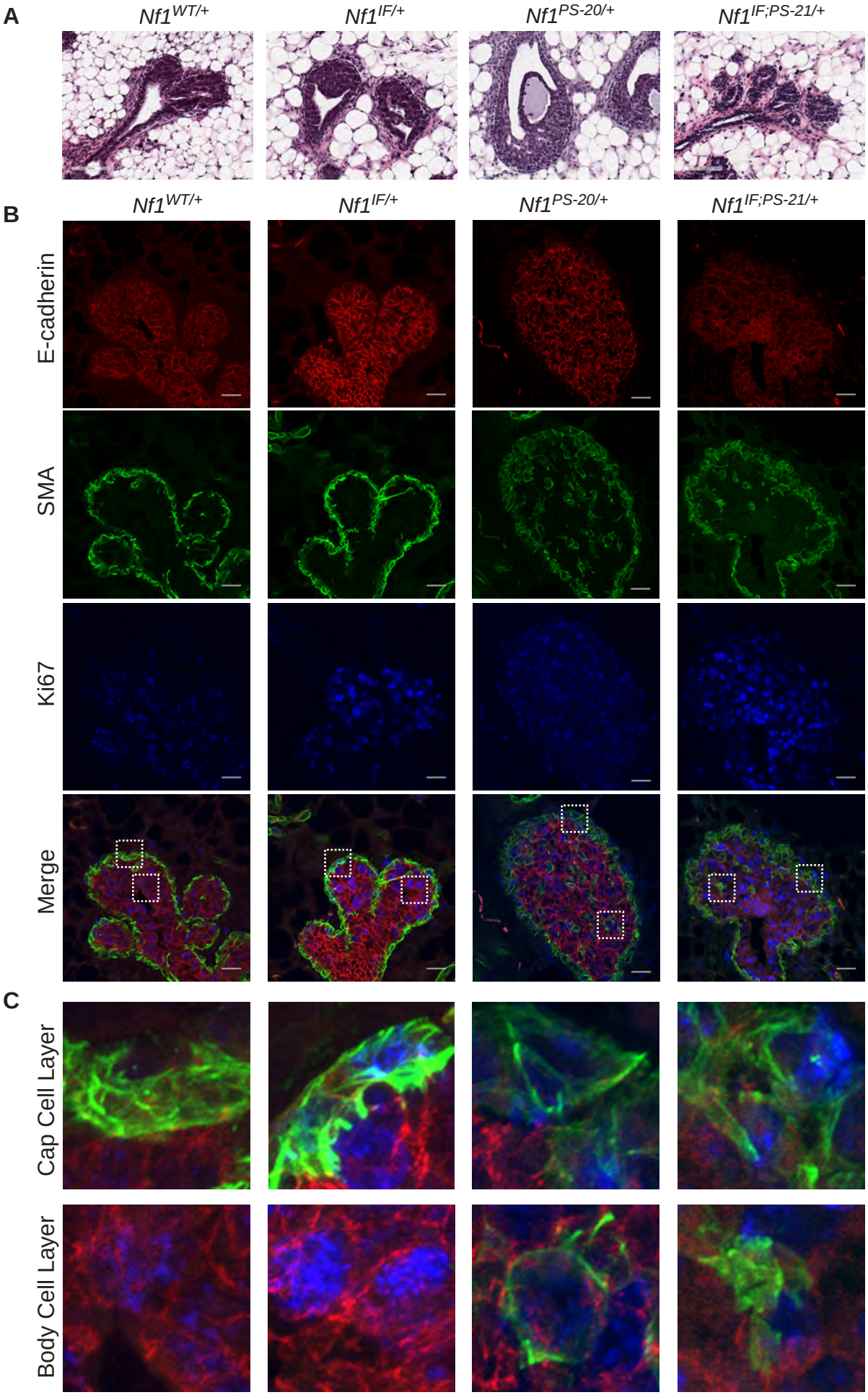

A
Nf1WT/+
Nf1IF/+
Nf1PS-20/+
Nf1IF;PS-21/+
B
Nf1WT/+
Nf1IF/+
Nf1PS-20/+
Nf1IF;PS-21/+
E-cadherin
SMA
Ki67
Merge
C
Cap Cell Layer
Body Cell Layer

Figure 3

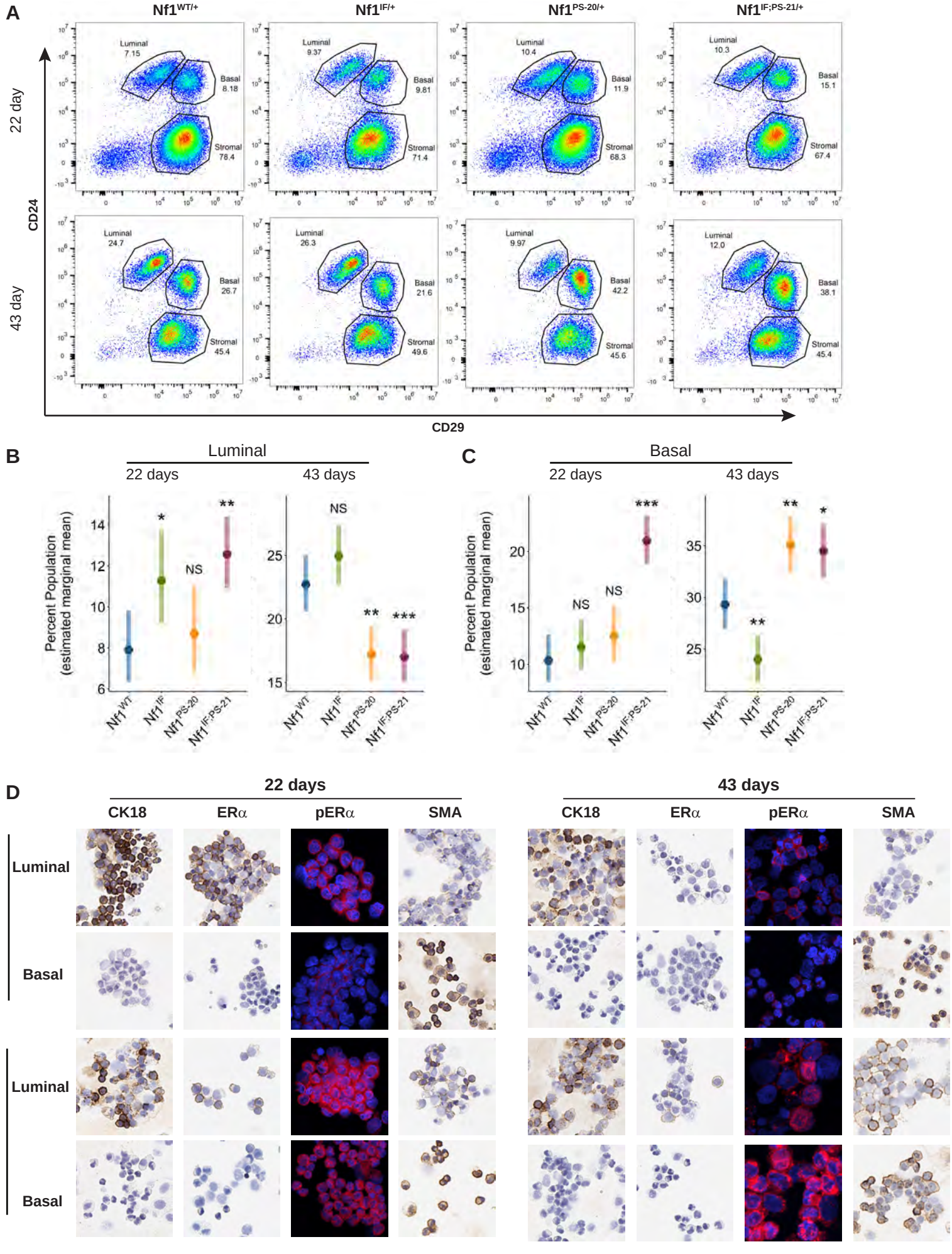

A
Nf1WT/+
Nf1IF/+
Nf1PS-20/+
Nf1IF;PS-21/+
22 day
43 day
CD24
CD29
Luminal
Basal
Stromal
7.15
8.18
78.4
9.37
9.81
71.4
10.4
11.9
68.3
10.3
15.1
67.4
24.7
26.7
45.4
26.3
21.6
49.6
9.97
42.2
45.6
12.0
38.1
45.4
B
Luminal
22 days
43 days
Percent Population (estimated marginal mean)
C
Basal
D
22 days
43 days
CK18
ERα
pERα
SMA
Nf1WT/+
Nf1IF;PS-21/+
Luminal
Basal

Figure 4

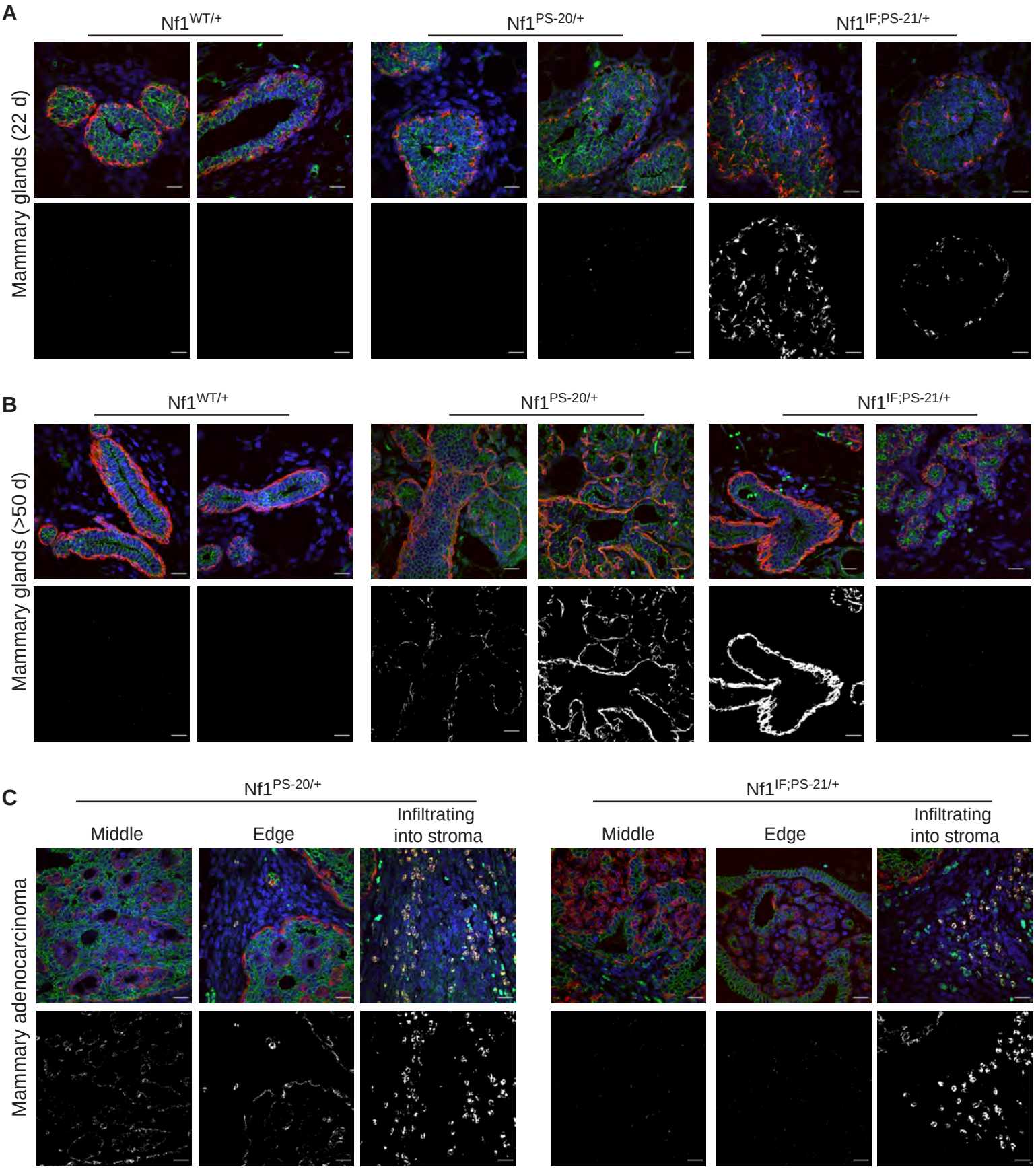
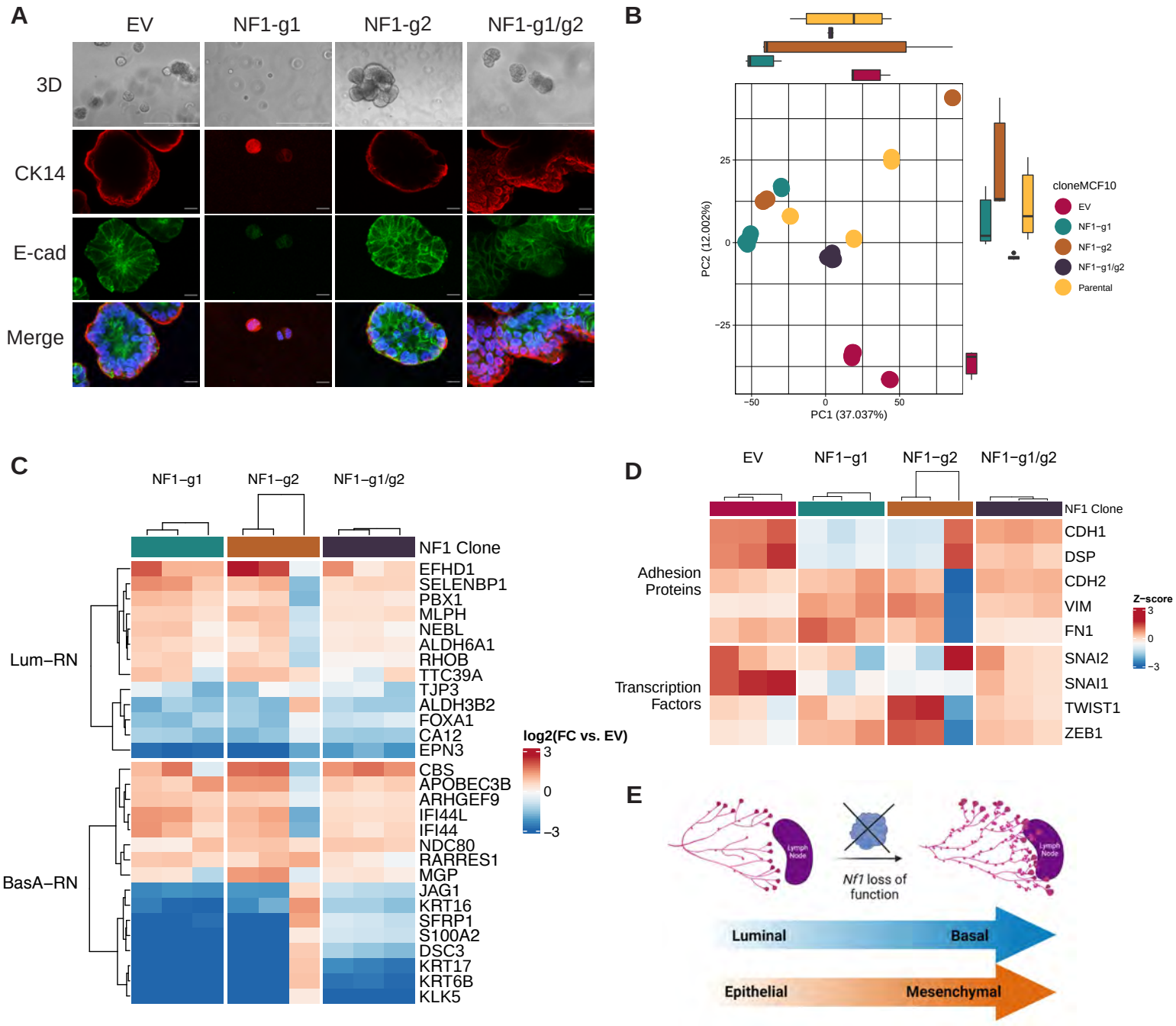

A
Nf1WT/+
Nf1PS-20/+
Nf1IF;PS-21/+
Mammary glands (22 d)
B
Nf1WT/+
Nf1PS-20/+
Nf1IF;PS-21/+
Mammary glands (>50 d)
C
Nf1PS-20/+
Nf1IF;PS-21/+
Middle
Edge
Infiltrating into stroma
Middle
Edge
Infiltrating into stroma
Mammary adenocarcinoma

Figure 5


A
EV
NF1-g1
NF1-g2
NF1-g1/g2
3D
CK14
E-cad
Merge
B
PC2 (12.002%)
PC1 (37.037%)
cloneMCF10
EV
NF1−g1
NF1−g2
NF1−g1/g2
Parental
C
NF1−g1
NF1−g2
NF1−g1/g2
NF1 Clone
Lum−RN
BasA−RN
EFHD1
SELENBP1
PBX1
MLPH
NEBL
ALDH6A1
RHOB
TTC39A
TJP3
ALDH3B2
FOXA1
CA12
EPN3
CBS
APOBEC3B
ARHGEF9
IFI44L
IFI44
NDC80
RARRES1
MGP
JAG1
KRT16
SFRP1
S100A2
DSC3
KRT17
KRT6B
KLK5
log2(FC vs. EV)
D
EV
NF1−g1
NF1−g2
NF1−g1/g2
NF1 Clone
Adhesion Proteins
Transcription Factors
CDH1
DSP
CDH2
VIM
FN1
SNAI2
SNAI1
TWIST1
ZEB1
Z−score
E
Lymph Node
Nf1 loss of function
Luminal
Basal
Epithelial
Mesenchymal

VAN ANDEL RESEARCH INSTITUTE
Opportunity Number: W81XWH2110759 RPPR

Name of Individual: STEENSMA, MATTHEW

Appointments:	Associate Professor	Van Andel Research Institute	United States
	Associate Professor	Michigan State University	United States
	Physician	Spectrum Heath	United States

Commons ID: STEENSMM

Other Support – Project/Proposal:

ACTIVE

Title: Targeting P53-Associated Therapy Resistance in NF1-Related MPNSTs

Major Goals: The major goal of this project is to show NF1 deficiency promotes breast cancer initiation and endocrine resistance through both direct and indirect mechanisms of deregulated ER signaling and altered mammary progenitor differentiation

Specific Aims: 1) Aim 1: To establish the indirect and direct mechanisms of NF1-ER signaling in normal mammary cells and breast cancer cells. Aim 2: To determine the effect of NF1 deficiency on ER signaling in mammary epithelial progenitor and tumor initiating populations. Aim 3: To determine the NF1 structural variants in sporadic breast cancer and efficacy of NF1 and ER inhibition.

Status of Support: Active

Project Number: W81XWH-18-NFRP-NIA

Name of PD/PI: Steensma, Matthew

Source of Support: Department of Defense

Agency Contact: Anna Tschiffely email: anna.e.tschiffely.ctr@mail.mil

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 07/15/2019 – 07/14/2022

Total Award Amount (including Indirect Costs):

Person Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2022	2.88 CM

Overlap: None

Title: Alternative NF1 isoforms in RAS deregulation and breast cancer progression

Major Goals: The major goal of this project 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

Specific Aims: Aim 1: Determine Nf1 isoform expression during Nf1-deficient breast cancer progression. Aim 2: Determine Nf1 isoform expression in endocrine-resistant Nf1-deficient breast cancers. Aim 3: Identify NF1 isoform expression in human breast cancers

Status of Support: Active

Project Number: W81XWH2110224

Name of PD/PI: Steensma, Matthew

Source of Support: Department of Defense

Agency Contact: Anna Tschiffely email: anna.e.tschiffely.ctr@mail.mil

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 06/15/2021 –

06/14/2023 Total Award Amount (including Indirect Costs):

Person Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2023	0.48 CM

Overlap: None

Title: The role of NF1 in mammary development, breast cancer and endocrine resistance

Major Goals: We hypothesize that NF1 deficiency promotes breast cancer and endocrine resistance in NF-related and sporadic breast cancer by altered AP-1 and ER signaling and chromatin remodeling

Specific Aims: 1) Determine the effect of NF1 deficiency on mammary gland development and breast cancer initiation, 2) Examine the impact of NF1 deficiency on chromatin dynamics and endocrine resistance, 3) Determine the efficacy of SERD and/or MEK inhibition in preventing NF1-deficient breast cancer and NF1-mediated chromatin remodeling.

Status of Support: Active

Project Number: W81XWH-21-1-0759 [THIS PROJECT]

Name of PD/PI: Steensma, Matthew

Source of Support: Department of Defense

Agency Contact: Anna Tschiffely email: anna.e.tschiffely.ctr@mail.mil

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 08/15/2021 – 08/14/2024

Total Award Amount (including Indirect Costs):

Person Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2023	0.70 CM
2024	0.70 CM

Overlap: None

Title: MET: an important therapeutic target for aggressive breast cancer

Major Goals: The major goal of this project is to evaluate how the Met oncogene influences tumor cell invasion

Specific Aims: 1) Determine the molecular and metabolic mechanisms of Met-induced cell motility; 2) Development of novel MRI CEST based breast cancer molecular imaging; 3) Determine the unique roles of Met and ErbB2 in tumorigenic growth and metastasis

Status of Support: Active

Project Number: BCRF-21-162

Name of PD/PI: Tsarfaty, Ilan (Contact); Graveel, Carrie

Source of Support: Breast Cancer Research Foundation

Agency Contact: Margaret (Peg) Mastrianni, The Breast Cancer Research Foundation, 60 East 56th St., 8th Floor, New York, NY 10022

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 10/01/2021 – 09/30/2022

Total Award Amount (including Indirect Costs): Person

Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2022	0.00 CM

Overlap: None

PENDING
None

RECENTLY COMPLETED
Title: Distinct Kinome Alterations in NF1- related Breast Cancer

Major Goals: The major goal of this project is to understand how NF1-related and sporadic breast cancers.

Project Number: N/A

Name of PD/PI: Steensma, Matthew

Source of Support: Spectrum Health

Agency Contact: Heather Zac, Spectrum Health Hospital Group, Office of Research Administration, 100 Michigan St NE, MC038, Grand Rapids, MI 49503

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 01/01/2017 – 12/31/2018

Total Award Amount (including Indirect Costs):

Title: SPRA: Graveel – Investigating the efficacy of SERDs in RAS-deregulated breast cancer

Major Goals: The major goal of this project is to determine the efficacy of SERDs in NF1-related breast cancer progression.

Specific Aims: 1) Determine the efficacy of novel SERDs in ER+ breast cancer, 2) Determine the efficacy of ER modulation in NF1-deficient breast cancers, and 3) Determine the effect of NF1-deficiency on ER signaling in normal mammary and breast cancer.

Project Number: N/A

Name of PD/PI: Steensma, Matthew

Source of Support: Genentech

Agency Contact: Ciara Metcalfe, 1 DNA Way, South San Francisco, CA, 94080

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 06/01/2018 – 12/31/2021

Total Award Amount (including Indirect Costs):

Title: SPRA: NF1 us a breast cancer driver in specific NF-related breast cancers

Major Goals: The major goal is to determine the pharmacodynamics and efficacy of a novel ERα-CIDE in ER+ breast cancer.Specific Aims: Specific Aim: Determine the pharmacodynamics and efficacy of a novel ERα-CIDE in ER+ breast cancer.

Project Number: N/A

Name of PD/PI: Steensma, Matthew

Source of Support: Genentech

Name of Individual: STEENSMA, MATTHEW
Commons ID: STEENSMM

Agency Contact: Ingrid E. Wentz, MD, PhD, 1 DNA Way, M/S 40, South San Francisco, CA 94080

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 04/19/2019 - 04/19/2021

Total Award Amount (including Indirect Costs):

Title: The Role of NF1 in estrogen signaling and breast cancer

Major Goals: The major goal of this project is to understand how NF1-related and sporadic breast cancers.

Specific Aims: Specific Aim 1: To establish the indirect and direct mechanisms of NF1-ER signaling in normal mammary cells and breast cancer cells. Specific Aim 2: To determine the effect of NF1 deficiency on ER signaling in mammary epithelial progenitor and tumor initiating populations. Specific Aim 3: To determine the effectiveness of RASand ER inhibition in breast cancer and mammary progenitor tumor initiating populations.

Project Number: None

Name of PD/PI: Steensma, Matthew

Source of Support: Van Andel Institute Internal Funding

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 11/27/2019 – 11/26/2020

to fund research for both NF1 and NF2. While NF has no cure and few treatments, researchers across the country and here in Michigan are working tirelessly to improve treatments and ultimately cure NF. Year by year they make discoveries that help enhance the health and quality of life of those affected.

Status of Support: Active

Primary Place of Performance: Van Andel Research Institute

Estimated Dollar Value of In-Kind Information:

Summary of In-Kind Contribution: **Eagles for Eric** - Eagles for Eric began as Birdies Beat Cancer, a golf outing, put on by friends and family to raise support for Eric's medical bills during his treatment for Osteosarcoma. After Eric passed away in May of 2016, the name was changed, and a second golf outing was hosted to raise money for his son, Arie's, future tuition. The organizational and party planning are now by partnered with Van Andel Institute to raise money for the research of Osteosarcoma.

Status of Support: Active

Primary Place of Performance: Van Andel Research Institute

Estimated Dollar Value of In-Kind Information:

Summary of In-Kind Contribution: **Bee Brave** - Throughout the last 15 years, Bee Brave has grown to become a community of like-minded individuals who are passionate about raising breast cancer awareness, supporting fighters, and honoring those whose battle has ended. Bee Brave is a haven of hope for those who need it, a home in which to share their courageous stories, as well as a place to celebrate the victories and triumphs over breast cancer. 100% of the money raised stays in Grand Rapids, Michigan.

Status of Support: Active

Primary Place of Performance: Van Andel Research Institute

Estimated Dollar Value of In-Kind Information:

Summary of In-Kind Contribution: **Outliers** - Pediatric and adult disorders that have no known cause but are expected to be driven by germline genetic variants.

Status of Support: Complete

Primary Place of Performance: Van Andel Research Institute

Estimated Dollar Value of In-Kind Information:

Summary of In-Kind Contribution: **Tempting Tables** - A Muskegon, Michigan based non-profit that raised money for breast cancer survivors, awareness, and research. The organization ended operations in 2020.

Status of Support: Complete

Primary Place of Performance: Van Andel Research Institute

Estimated Dollar Value of In-Kind Information:

Material Transfer Agreements:

Spectrum Health, UNITED STATES (VARI Ref: 3428)
Other: Joint Appointment Agreement between Dr. Matt Steensma and Spectrum Health; automatically renews for additional one year terms after the expiration date if not terminated.
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Effective: 1/1/2017-12/31/2022

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Effective: 2/21/2018

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Material Transfer Agreement - Receiving: MPNST cell lines (human and mouse derived) and chemicals for activating P53.
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Effective: 6/26/2019

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Effective: 6/27/2019

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Material Transfer Agreement - Receiving: MPNST patient-derived tumorgraft tissue and cell lines.
Effective: 10/14/2019

Dana Farber Cancer Institute, UNITED STATES (VARI Ref: 3086)

Material Transfer Agreement - Providing: Please reference record 1719 for background information. Steensma Lab provided materials to the Haigis Lab at Beth Israel Deaconess Medical Center via a UBMTA in 2016. Lab is moving to Dana Farber Cancer Institute and is requesting to utilize the KRASG13D mouse strain originally developed in Steensma Lab.
Effective: 7/14/2020

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Material Transfer Agreement - Providing: 12 de-identified human tissue samples from normal human subjects and neurofibromatosis patients. i.e., 6 of each group. rel 1030.
Effective: 4/11/2022

I, PD/PI or other senior/key personnel, certify that the statements within this current and pending document are true, current, complete and accurate to the best of my knowledge. I agree to update such disclosure at the request of the agency prior to the award of support and at any subsequent time the agency determines appropriate during the term of the award. I have been made aware of the requirements under Section 223(a)(1) of the William M. (Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021. I am aware that any false, fictitious, or fraudulent statements or claims may result in criminal, civil, or administrative penalties (U.S. Code, Title 18, Section 1001).

Signature/Date: _____

VAN ANDEL RESEARCH INSTITUTE
Opportunity Number: W81XWH2110759 RPPR

Name of Individual: GRAVEEL, CARRIE
Appointments: Senior Research Scientist Van Andel Research Institute United States
Commons ID: CARRIE.GRAVEEL

Other Support – Project/Proposal:

ACTIVE

Title: Targeting P53-Associated Therapy Resistance in NF1-Related MPNSTs

Major Goals: The major goal of this project is to show NF1 deficiency promotes breast cancer initiation and endocrine resistance through both direct and indirect mechanisms of deregulated ER signaling and altered mammary progenitor differentiation

Specific Aims: 1) Aim 1: To establish the indirect and direct mechanisms of NF1-ER signaling in normal mammary cells and breast cancer cells. Aim 2: To determine the effect of NF1 deficiency on ER signaling in mammary epithelial progenitor and tumor initiating populations. Aim 3: To determine the NF1 structural variants in sporadic breast cancer and efficacy of NF1 and ER inhibition.

Status of Support: Active

Project Number: W81XWH-18-NFRP-NIA

Name of PD/PI: Steensma, Matthew

Source of Support: Department of Defense

Agency Contact: Anna Tschiffely email: anna.e.tschiffely.ctr@mail.mil

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 07/15/2019 – 07/14/2022

Total Award Amount (including Indirect Costs):

Person Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2022	3.0 CM

Overlap: None

Title: Alternative NF1 isoforms in RAS deregulation and breast cancer progression

Major Goals: The major goal of this project 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

Specific Aims: Aim 1: Determine Nf1 isoform expression during Nf1-deficient breast cancer progression. Aim 2: Determine Nf1 isoform expression in endocrine-resistant Nf1-deficient breast cancers. Aim 3: Identify NF1 isoform expression in human breast cancers

Status of Support: Active

Project Number: W81XWH2110224

Name of PD/PI: Steensma, Matthew

Source of Support: Department of Defense

Agency Contact: Anna Tschiffely email: anna.e.tschiffely.ctr@mail.mil

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 06/15/2021 – 06/14/2023

Total Award Amount (including Indirect Costs):

Person Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2023	1.2 CM

Overlap: None

Title: The role of NF1 in mammary development, breast cancer and endocrine resistance

Major Goals: We hypothesize that NF1 deficiency promotes breast cancer and endocrine resistance in NF-related and sporadic breast cancer by altered AP-1 and ER signaling and chromatin remodeling

Specific Aims: 1) Determine the effect of NF1 deficiency on mammary gland development and breast cancer initiation, 2) Examine the impact of NF1 deficiency on chromatin dynamics and endocrine resistance, 3) Determine the efficacy of SERD and/or MEK inhibition in preventing NF1-deficient breast cancer and NF1-mediated chromatin remodeling.

Status of Support: Active

Project Number: W81XWH-21-1-0759 [THIS PROJECT]

Name of PD/PI: Steensma, Matthew

Source of Support: Department of Defense

Agency Contact: Anna Tschiffely email: anna.e.tschiffely.ctr@mail.mil

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 08/15/2021 – 08/14/2024

Total Award Amount (including Indirect Costs):

Person Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2023	4.2 CM
2024	4.2 CM

Overlap: None

Title: MET: an important therapeutic target for aggressive breast cancer

Major Goals: The major goal of this project is to evaluate how the Met oncogene influences tumor cell invasion

Specific Aims: 1) Determine the molecular and metabolic mechanisms of Met-induced cell motility; 2) Development of novel MRI CEST based breast cancer molecular imaging; 3) Determine the unique roles of Met and ErbB2 in tumorigenic growth and metastasis

Status of Support: Active

Project Number: BCRF-21-162

Name of PD/PI: Tsarfaty, Ilan (Contact); Graveel, Carrie

Source of Support: Breast Cancer Research Foundation

Agency Contact: Margaret (Peg) Mastrianni, The Breast Cancer Research Foundation, 60 East 56th St., 8th Floor, New York, NY 10022

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 10/01/2021 – 09/30/2022

Total Award Amount (including Indirect Costs):

Person Months (Calendar) per budget period:

Year (YYYY)	Person Months (##.##)
2022	2.4 CM

Overlap: None

PENDING
None

Title: Distinct Kinome Alterations in NF1- related Breast Cancer

Major Goals: The major goal of this project is to understand how NF1-related and sporadic breast cancers.

Project Number: N/A

Name of PD/PI: Steensma, Matthew

Source of Support: Spectrum Health

Agency Contact: Heather Zac, Spectrum Health Hospital Group, Office of Research Administration, 100 Michigan St NE, MC038, Grand Rapids, MI 49503

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 01/01/2017 – 12/31/2018

Total Award Amount (including Indirect Costs):

Title: SPRA: Graveel – Investigating the efficacy of SERDs in RAS-deregulated breast cancer

Major Goals: The major goal of this project is to determine the efficacy of SERDs in NF1-related breast cancer progression.

Specific Aims: 1) Determine the efficacy of novel SERDs in ER+ breast cancer, 2) Determine the efficacy of ER modulation in NF1-deficient breast cancers, and 3) Determine the effect of NF1-deficiency on ER signaling in normal mammary and breast cancer.

Project Number: N/A

Name of PD/PI: Steensma, Matthew

Source of Support: Genentech

Agency Contact: Ciara Metcalfe, 1 DNA Way, South San Francisco, CA, 94080

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 06/01/2018 – 12/31/2021

Total Award Amount (including Indirect Costs):

Title: SPRA: NF1 us a breast cancer driver in specific NF-related breast cancers

Major Goals: The major goal is to determine the pharmacodynamics and efficacy of a novel ERα-CIDE in ER+ breast cancer.Specific Aims: Specific Aim: Determine the pharmacodynamics and efficacy of a novel ERα-CIDE in ER+ breast cancer.

Project Number: N/A

Name of PD/PI: Steensma, Matthew

Source of Support: Genentech

Agency Contact: Ingrid E. Wentz, MD, PhD, 1 DNA Way, M/S 40, South San Francisco, CA 94080

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 04/19/2019 - 04/19/2021

Name of Individual: GRAVEEL, CARRIE
Commons ID: CARRIE.GRAVEEL

Total Award Amount (including Indirect Costs):

Title: The Role of NF1 in estrogen signaling and breast cancer

Major Goals: The major goal of this project is to understand how NF1-related and sporadic breast cancers.

Specific Aims: Specific Aim 1: To establish the indirect and direct mechanisms of NF1-ER signaling in normal mammary cells and breast cancer cells. Specific Aim 2: To determine the effect of NF1 deficiency on ER signaling in mammary epithelial progenitor and tumor initiating populations. Specific Aim 3: To determine the effectiveness of RASand ER inhibition in breast cancer and mammary progenitor tumor initiating populations.

Project Number: None

Name of PD/PI: Steensma, Matthew

Source of Support: Van Andel Institute Internal Funding

Primary Place of Performance: Van Andel Research Institute

Project/Proposal Start and End Date: 11/27/2019 – 11/26/2020

Total Award Amount (including Indirect Costs):

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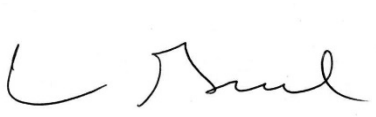

Signature/Date: _____