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GENETIC AND FUNCTIONAL ANALYSIS OF RAT MAMMARY CANCER
SUSCEPTIBILITY

By

Emily L. Duderstadt
B.S. Wittenberg University 2016
M.S. University of Louisville 2018

A Dissertation
Submitted to the Faculty of the
School of Medicine
of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy in Biochemistry and Molecular Genetics

Department of Biochemistry and Molecular Genetics
University of Louisville
Louisville, KY

May 2021

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A dissertation approved on

April 21, 2021

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I would like to thank my parents, Chuck and Jackie, for investing in my education since my birth. I would not be here without their willingness to sacrifice a comfortable life for themselves so I could have a better educational environment. They also instilled in me a strong work ethic and academic drive. I would like to thank my brother Nathan, who as my “twin” has been my companion and supporter through every shared milestone since pre-school. I would like to thank my best friend Lisa Green, who has been there for half of my life and supported me on our shared career path. Additionally, I would like to express an appreciation for my hound dog Lena, who has been a source of comfort to me through all of the lonely and terrible times in college and graduate school.

ABSTRACT

GENETIC AND FUNCTIONAL ANALYSIS OF RAT MAMMARY CANCER
SUSCEPTIBILITY

Emily L. Duderstadt

April 21, 2021

Breast cancer genetic susceptibility is modulated by high to low penetrance risk alleles. Population frequencies of high and moderate penetrant predisposing mutations are rare and responsible for a small percentage of population cases. Most genetic variation in breast cancer susceptibility is low penetrance common variants where causal genes and risk-associated mechanisms are largely unknown. Comparative genetics uses animal models to isolate and mechanistically evaluate genetic susceptibility to human disease. Rat strains have different susceptibility phenotypes to chemical-induced carcinogenesis. Selective breeding and genetic linkage analysis using a 7,12-dimethylbenz(a)anthracene (DMBA) rat mammary carcinogenesis model have identified *Mammary carcinoma susceptibility* quantitative trait loci (*Mcs* QTLs). Research completed for this dissertation focused on *Mcs3* and *Mcs1b* QTLs. I found elevated mammary gland expression of *Mcs3*-nominated and established breast cancer gene *p21-activated kinase (Pak1)* in response to DMBA administration. This highlighted a potential role for this gene in disease initiation. *Mcs3* was previously mapped to a 29.4 Mb region on rat chromosome 1 using WF.COP

congenic lines. I developed my own congenic lines and fine mapped this locus to 7.2 Mb, discovered pleiotropic effects of *Mcs3* on mammary tumor multiplicity and body mass, and identified candidate breast cancer and body mass genes/variants in orthologous human regions. Rat *Mcs1b* maps to 1.8 Mb on rat chromosome 2 and is orthologous to human breast cancer risk locus *5q11.2*. An *Mcs1b* and *5q11.2*-nominated gene is *MIER family member 3 (MIER3)*. I used an established rat *Mcs1b* congenic model to study *in situ Mier3* mammary gland expression and found most expression in luminal epithelial cells. Expression of *Mier3* doubled upon DMBA administration and increased along a precursor-carcinoma disease progression model. *Mier3* expression was significantly enhanced in embryonic mammary buds, neonatal, and pubertal mammary glands, with significant drop-off at adulthood. This implicates *MIER3* as a breast cancer gene that may modulate disease susceptibility during early periods of female breast development. I also provide *MIER3* mechanistic insight by identifying interactions of *MIER3* with nuclear proteins involved in transcriptional regulation and heterochromatin formation. Thus, *MIER3* may function within a novel HDAC1/2 co-repressor complex like NuRD, Sin3A, CoREST, and family member *MIER1*.

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CHAPTER I

BREAST CANCER INTRODUCTION

Breast Cancer Statistics

Breast cancer is the most frequently diagnosed cancer among women, accounting for 30% of all diagnosed cancers. This is significant as the second most common cancer in women is lung cancer, with 13% of cancer incidences [1]. In the U.S. alone in 2021 there are projected to be 281,550 new cases and an estimated 43,600 deaths due to breast cancer [1]. Although the breast cancer death rate has dropped by 41% since 1989, it is still the second leading cause of cancer-related deaths in women at 15% of cancer mortalities [1]. Over the last two decades, breast cancer incidence rates have increased by about 0.5% per year, which is partly attributable to lifestyle risk factors such as older age at first birth and increased body weight [2]. This highlights a pressing need to identify and investigate further genetic and molecular susceptibility factors to aid in early screening and prevention strategies.

Breast Cancer Risk Factors

Breast cancer is a highly complex disease comprised of environmental and genetic components, as well as the interplay between them. A woman's individual disease risk varies depending on her possession of underlying factors that modulate overall susceptibility. Risk factors attributable to breast disease initiation can be divided into two

groups, intrinsic and extrinsic factors. Intrinsic risk factors are inherent and cannot be changed by outside intervention. Extrinsic factors can be modified to a certain degree within individuals and include lifestyle choices and environmental influences. Some of the major risk factors are outlined below. This is not an exhaustive list, but serves to illustrate the broad range of risk factors that influence breast cancer susceptibility.

Intrinsic risk factors:

Sex:

Biological sex is a major risk factor for developing breast cancer, as women have 100 times greater disease incidence than men [3]. However, despite diagnosis predominantly in women, the number of male breast cancer cases is increasing, which is thought to be attributable to obesity and longer life expectancy [4, 5].

Age:

Breast cancer incidence is highly related to increasing age, with 80% of diagnosis coming from women aged 50 and over [6]. The number of breast cancer cases is very low in women younger than 30 (<25 cases per 100,000), after which there is a steady linear increase and a plateau around the age of 80 (~500 cases per 100,000) [7]. Hormone responsiveness is also stratified by age. Estrogen receptor negative (ER-) tumors occur more frequently until around 50 years of age, *i.e.*, the approximate average age at menopause, followed by a greater incidence of ER+ tumors in women after menopause [8].

Endogenous estrogen levels:

High levels of estrogen-related hormones (total estradiol, free estradiol, estrone, estrone sulfate) are a well-defined breast cancer risk factor. Increasing estrogen levels especially enhance breast cancer risk in post-menopausal women and is correlated with increased incidence of ER+ breast tumors [9, 10]. This phenomenon has been demonstrated using aromatase inhibitors (AI), which block the conversion of androgens to estrogens and lowers blood estrogen levels. Phase III trials have demonstrated a 50-65% reduction in invasive breast cancer in post-menopausal women treated with AI (which ones) at high risk of breast cancer compared to placebo [11, 12]. Although estrogens confer higher risk in post-menopausal women, there is still a critical role of estrogens in the etiology of pre-menopausal breast cancer [13, 14].

Reproductive factors:

Exposure to and changes in endogenous sex hormones can influence breast cancer risk. Early menarche (less than 12 years of age) is associated with higher risk as compared to menses onset after age 15 [15]. Every two-year delay of menarche reduces disease risk by 10% [15-17]. At the opposite end of reproduction, breast cancer risk increases 3% for every one year increase in the age at menopause [18]. These higher risks of disease occurrence are thought to be due to the duration of menstrual cycles over a woman's lifetime, with prolonged estrogen exposure resulting from early menarche and delayed menopause. The risk of breast cancer is also increased in nulliparous women or women who have a first-time birth after age 30 [19, 20].

Genetic susceptibility:

Genetic predisposition to breast cancer can come from inherited familial susceptibility or *de novo* germline mutations. Cases of familial disease is a major risk factor as 12% of women diagnosed with breast cancer have at least one affected family member [21]. Women with one first-degree relative with breast cancer have a 1.75-fold higher risk of disease occurrence, which increases to 2.5-fold higher in women with two or more first-degree relatives with breast cancer [22].

Mammographic density:

A mammography assesses the radiographic appearance of breast tissue, which varies depending on the ratio of fat, connective tissue, and epithelial tissue (radiographically most dense). Women with more than 75% of their breast tissue radiographically defined as dense have a 5-fold greater risk of breast cancer development than women with only 5% of tissue classified as dense [23]. Thinner and younger women, as well as both pre- and post-menopausal nulliparous women, generally have increased breast density and therefore may have increased risk of breast cancer [24].

Extrinsic Risk Factors:***Diet:***

The modern human diet contains a variety of natural and chemical carcinogens which can lead to DNA damage and cancer initiating events [25, 26]. Many of these come from the processing and preservation of food, especially the preparation of meat [27]. A diet low in bread and fruits, but high in meat, fish, butter, and other animal fats is associated

with increased disease risk [28]. A major dietary risk factor is the excess intake of saturated fat, which is highly concerning for a western diet containing too much saturated fats [29, 30]. This risk may be attributable to an increase in circulating estradiol due to saturated fats [31]. It has been demonstrated that implementing a low-fat diet in post-menopausal women, after a primary breast cancer surgical procedure, significantly reduced the risk of relapse [26]. Additionally, a diet rich in fiber, omega-3 polyunsaturated fatty acids, fruits, and vegetables seems to have a protective effect against breast cancer [32-34].

Alcohol consumption:

There is a positive association between alcohol intake and breast cancer risk. Ingesting 35-44 grams of alcohol per day can cause a 32% increase in risk, with each additional 10 grams resulting in a 7.1% increase [35]. The main breast cancer specific risk is the effect of alcohol on estrogen metabolism, which results in elevated serum levels of estrogen-related hormones and induction of ER pathways in pre- and post-menopausal women [36-38]. Another critical pathogenic mechanism is through the action of the alcohol metabolite acetaldehyde, which is an established carcinogen [39].

Exercise:

Low levels of physical activity are associated with breast cancer risk at any age. There is a 25% average reduction in breast cancer risk across 73 studies that compared the most active to least physically active women [40]. Physical activity during adolescence and young adulthood significantly reduces breast cancer risk, as there is a 3% reduction in risk for each one-hour increase in recreational activity per week [41]. Associations for reduced

risk are strongest for activity that is minimally moderate in intensity and is sustained over a lifetime or done after menopause [42]. Mechanisms of reduced risk are thought to stem from reduction in circulating sex hormones which lessens cumulative exposure and activation of hormonal pathways, as well as modulation of adipocytokines and inflammation [43].

Obesity:

The risk of breast cancer in association with obesity appears to be dependent on a woman's menopausal status. In post-menopausal women obesity positively associates with breast cancer risk, with an 8% risk increase for each 5 kg of weight gain since the lowest adult weight [44]. This is largely thought to be due to the greater amounts of adipose tissue in obese women which serves as an extragonadal source of estrogen, resulting in continued estrogen exposure despite loss of menstrual cycling [45, 46]. In contrast, obesity is associated with a decreased risk of breast cancer in pre-menopausal women, although the underlying mechanism is not understood [47, 48].

Smoking:

The relationship between smoking and breast cancer risk is not definitive, probably due to a genetic and environmental interplay on risk association that has been demonstrated and low carcinogen dose [49, 50]. Some studies have indicated early age of smoking initiation and long duration of smoking years can increase breast cancer risk 15% to 40% [50]. Of the thousands of chemicals in cigarette smoke, 69 are carcinogenic and 20 of these specific mammary carcinogens [51]. Mammary tissue can take up and bioactivate

polycyclic aromatic hydrocarbons and N-nitrosamines from cigarette smoke, eventually resulting in formation of DNA adducts [49, 52, 53].

Environmental pollutants:

There are two main environmental pollutants that are associated with breast cancer risk, both of which are lipophilic and accumulate in the high adipose content of breast tissue – polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). PAHs are a by-product of combustion and are prevalent in grilled foods, air pollution, vehicle exhaust, and cigarette smoke [51, 54]. Several PAHs, including one of the more widely recognized PAHs benzo(a)pyrene, are mammary mutagens that form DNA adducts that induce DNA damage and repair responses [55]. PCBs were contained in lubricants, liquid sealants, flame retardants, and electrical insulators until banned in the U.S. in 1979. They remain pervasive in the environment, and the main route of PCB exposure is through ingestion of contaminated fish and animal fats. PCBs are found in most samples of human breast milk [56]. PCBs are highly relevant to breast cancer risk since metabolites serve as endocrine disrupting chemicals. PCBs also induce DNA damage, which can lead to carcinogenesis [56].

Genetic Factors of Breast Cancer Risk

Among women diagnosed with breast cancer, 10-20% already have a family history of disease [57, 58]. This clustering of breast cancer cases within families provides evidence there is a genetic predisposition underlying cancer development, though it is difficult to parse out genetic and environmental factors in disease epidemiology. The genetic

contribution has been estimated using twin studies that compare disease occurrence in monozygotic (genetically identical) and dizygotic (share half of their segregating genes) twin pairs [59]. Monozygotic twins share a greater disease concordance than dizygotic twins, meaning a monozygotic twin has a higher risk of developing breast cancer if their twin is affected, demonstrating there are powerful hereditary factors contributing to breast cancer susceptibility [59-62]. Analysis of familial clustering has estimated the heritability of breast cancer to be 73% within families [63], while 10-30% of non-familial disease susceptibility has been attributed to genetic factors [59, 61, 62, 64]. Since recognizing the large genetic component to the origin of breast disease, it became essential to identify inherited susceptibility factors and their biological mechanism.

Breast cancer risk genes can be divided into three classes based on allele frequency and conferred risk. The first class is comprised of high penetrance risk alleles such as *BRCA1*, *BRCA2*, *TP53*, *STK11*, and *CDH1* [64]. Risk variants in these genes are very rare in the population but each individual allele has a strong effect on breast cancer risk [65]. Early genetic studies using linkage analysis within families displaying aggregation of breast cancer risk first identified *BRCA1* and *BRCA2* as highly penetrant susceptibility genes [66, 67]. These cause breast cancer incidences of 65% and 45% in carrier women of *BRCA1* and *BRCA2* mutations, respectively, by the age of 70 [68]. Further linkage studies and candidate gene approaches in breast cancer families that do not segregate *BRCA1* or *BRCA2* mutations have identified additional genes. These studies were successful in accounting for up to 25% of the heritability of familial breast cancer [65, 69]; however, they do little in explaining genetic factors behind sporadic disease development, accounting for only up to 5% of heritability [65]. Since the search for new highly penetrant

genes eventually proved exhaustive, the unexplained genetic risk provided evidence of existence of other undetected susceptibility genes.

Moderate risk alleles make up the second class of breast cancer heritability factors, which are variants with a rare population frequency and a moderate effect on disease risk. Genes in this class of breast cancer risk alleles include *PTEN*, *CHECK2*, *PALB2*, *ATM*, *BRIP1*, *BARD1*, *ABRAXAS* [64]. These predisposing genes have been identified through linkage analysis and candidate gene approaches, which used targeted sequencing of genes known to interact with BRCA1 and BRCA2 or DNA repair pathway genes, followed by further evaluation in case-control association studies [63]. Other moderate risk alleles have been identified using linkage analysis in families with rare syndromes that include breast malignancies [70]. Similar to the high penetrance mutations, these moderately penetrant alleles account for very little non-familial disease risk and contribute less than 3% of the heritable familial risk [71]. High penetrance and moderate penetrance alleles together account for no more than 25% of familial breast cancer risk [69, 72], leaving the majority of genetic susceptibility unidentified.

The remaining fraction of breast cancer genetic risk is thought to be driven by low-penetrance alleles, which make up the third class of breast cancer risk alleles [73, 74]. Variants in this class are very common, each affecting >5% of the population, but individually have weak associations with risk [75]. It is estimated that the collective effects of these low penetrance risk alleles on population genetic risk exceeds that of highly penetrant genes [76, 77]. In addition to their combined effect on sporadic disease development, low-risk variants are thought to explain part of the residual genetic component of carriers of high-risk alleles by interacting multiplicatively on overall

susceptibility [78, 79]. Common low-penetrance alleles were initially identified by case-control genetic association studies targeting candidate genes known to be involved in carcinogenesis [80]. This approach is biased because it relies on prior knowledge of the genes and types of causative polymorphisms and it ignores uncharacterized genes [75]. The first genome-wide association study (GWAS) of breast cancer was published in 2007 and GWAS has since become the standard approach to identifying risk-associated variants and low-penetrance alleles [64]. In these studies, several hundred thousand SNPs are genotyped across very large study populations, allowing for association with disease risk to be conferred without assumption of biological function or location [70, 81]. As the number of GWAS have grown, meta-analysis combining multiple studies have become important in identifying additional susceptibility loci. To date, more than 180 breast cancer susceptibility loci have been identified [82]. There is now a great need to identify causal variants in these loci and biological mechanism behind disease susceptibility.

It is estimated that the collective common low penetrance risk variants identified by GWAS only account for 18% of familial breast cancer risk, and most of these reported associations have failed to be confirmed in validation studies [83, 84]. The remaining heritability from low penetrance alleles may remain unidentified due to limitations in GWAS. There were high levels of false-positives in initial reports because investigators failed to use stringent statistical evaluation to identify true risk loci and variants in which the probability of an association is small [83, 85]. A Bonferroni corrected p-value of less than 10^{-7} has been recommended as necessary to reduce the rate of false-positives, while other studies utilize a very stringent criteria of $p < 10^{-8}$ [85-87]. From this increased stringency has risen the idea that this increases the rate of false-negatives, meaning SNPs

associated with disease risk are missed due to too conservative of statistical methods in evaluating GWAS [88]. Thus, parsing out true risk-associated alleles and variants from GWAS proves difficult without complementary studies of genetic risk. Additionally, GWAS use “tag-SNPs” to tag common variation across the genome, but these only serve as a marker of a disease-associated region [70]. This is a result of linkage disequilibrium (LD), where numerous SNPs in a region of low recombination are inherited together [63]. These LD blocks, or haplotype blocks, are bound by regions of high recombination [63]. This delimits the region and means the GWAS tag-SNP identifies that LD block as associated with a disease phenotype, but leaves the true causal variant unknown [70]. Fine mapping of these LD blocks is then necessary to obtain a complete list of variants in the region, followed by genotyping of all SNPs in LD with the tag-SNP across large case-control studies to identify candidate causal variants based on the strongest disease-associations [63, 70].

GWA studies by design are capable of identifying common variants, but it is estimated a portion of the unidentified genetic risk of breast cancer stems from its inability to interrogate rare variants conferring moderate risks since these SNPs are not tagged or well represented in population-based studies [89]. The increase in the scale of next generation sequencing (NGS) and decline in sequencing cost has elevated this platform as the newest route for discovery of both rare and common breast cancer-associated variants [90, 91]. When investigating rare risk alleles there is reduced statistical power, requiring filtering of studies or sequencing of a large number of cases and controls. Some of these strategies include initially sequencing within cases of familial aggregation, selecting candidate genes to sequence based on pathways or disease implications, or filtering based

on consequences of variation on translation and protein function [63, 92]. Following discovery, variants can be validated in larger cohorts that contain controls and patients with sporadic and familial breast cancer [93].

Although high and moderate penetrant alleles have deleterious effects for familial breast cancer patients, the majority of the heritability remains unexplained in these families and do not provide insight into the risk of sporadic disease. The major genetic component of breast cancer most likely follows a polygenic model, where the aggregation of low penetrant alleles alters disease susceptibility in sporadic cases and can further modulate familial disease-risk through interaction with higher penetrant alleles. The genetic architecture of breast cancer, comprised of the three classes of genetic susceptibility and their minor allele frequency, is depicted in Figure 1. High and moderate penetrant alleles are typically found in coding regions of genes, making the identification of the causative gene clear and usually resulting in protein truncation due to nonsense mutations, frameshift insertions or deletions, or variants interrupting splice sites [63]. However, low penetrance alleles are generally found in non-coding or intergenic regions, which makes identifying the causative gene more difficult since the effect is mainly on regulation of gene expression. These risk alleles may affect promoter function, modulate binding of transcription factors, or reside at distal regulatory elements such as enhancers and affect gene regulation through complex chromatin arrangements [70]. In continuing the discovery of unexplained genetic factors behind breast cancer susceptibility, there is a dual challenge in identifying causative variants within susceptibility loci, as well as parsing a biological mechanism behind the associated risk.

Figure 1: Architecture of genetic susceptibility to breast cancer. Adapted from Ghousaini et al. (2013) [70]. Some of the major genetic loci identified for breast cancer risk are shown in this figure. Breast cancer risk genes are subdivided into classes by allele frequency and level of risk.

Timepoints of Altered Environmental and Genetic Susceptibility

There are susceptibility timepoints when genetic risk factors and environmental perturbations overlap with morphological changes in breast tissue that result in increased lifetime risk of developing breast cancer [94-99]. These “windows of susceptibility” are timepoints characterized by an increased risk of cancer initiating events due to molecular alterations that drive tissue remodeling, which can have a role in breast cancer development later in life [94, 96, 98, 99]. Windows of susceptibility encompass developmental and reproductive time intervals that are each unique in their susceptibility response due to distinct transcriptomes, epigenetic state, tissue microenvironment, and signaling processes that comprise breast tissue and its biological function at that time [94, 96, 97, 100].

Breast cancer susceptibility during puberty:

The most susceptible structures in the female breast are terminal ductal lobular unit (TDLU) or Lobules type 1 (Lob 1), which become a milk-secreting population during lactation [100-102]. These structures contain undifferentiated epithelial cells with high proliferative activity that first appear during puberty and expand during this time period. It is notable that Lob 1 epithelial cells can be transformed to induce mammary cancer following in vitro exposure to the same chemical carcinogens administered in experimental mammary cancer models, such as N-methyl-nitrosourea and 9,10-dimethyl-1,2-benzanthracene [100, 103]. Lob 1 structures have also been shown to have the highest rate of DNA binding by carcinogen [104]. This suggests that chemical insults affect Lob 1 structures, and a mutagenic event is most likely to transform breast tissue during this susceptibility window that begins at puberty and extends until a first full-term pregnancy

[100, 104]. From studies like these, there arises a concern that inadvertent chemical exposure during this high risk window may drastically increase susceptibility to breast cancer since it is the terminal ductal structures that give rise to the majority of breast cancers [105-112]. Thus, it is crucial to understand the biological characteristics of Lob 1 as these are the major sites of cancer initiation events.

Breast cancer protection timepoints due to tissue differentiation:

Differentiation is an inhibitor of cancer development, which is clinically relevant as undifferentiated structures are more likely to develop aggressive carcinomas [113-115]. This has been demonstrated in the human breast, which attains maximum development and differentiation during pregnancy, resulting in parous women displaying the greatest proportion of differentiated lobules and lower sensitivity to carcinogens [100, 107, 116, 117]. As a result of differentiation, an early first full-term birth is the most effective method of breast cancer prevention, which can reduce a woman's lifetime risk up to 50%, with additional pregnancies increasing this protective effect compared to nulliparous women [116, 118, 119]. Breast cancer protection due to pregnancy is inversely related to the length of time between menarche and the first reproductive cycle, as breast cancer risk increases with increased time of exposure of undifferentiated tissue [119, 120]. This protective effect is extremely powerful as it persists in all subsequent ages and through menopause [119, 120]. Breast cancer protection due to tissue differentiation is also demonstrated in parous women who do develop breast cancer, as their lobular profiles match that of nulliparous women. This indicates these women harbor a defective response to differentiation signaling and do not achieve full differentiation during the reproductive cycle, and thus retain

susceptible cell types [121-123]. Understanding the molecular mechanisms behind mammary gland differentiation would thus provide valuable knowledge to develop novel approaches of breast cancer prevention.

The protective effect of pregnancy has been recapitulated in a rat model of mammary cancer. Rats administered a chemical carcinogen to induce mammary tumors are similarly protected by pregnancy as compared to nulliparous females [124-126]. Interestingly, merely replicating the hormonal exposures of the mammary gland during pregnancy in virgin females can induce protection from carcinogen-induced mammary tumor multiplicity [127-130]. Combinatorial treatments with estradiol and progesterone shortly before or after chemical carcinogen administration have been shown to mimic pregnancy by inducing mammary gland lobuloalveolar formation and inhibiting mammary tumor formation [128, 131]. It is assumed that the same molecular factors in the human breast and rat mammary gland are responsible for tissue differentiation that inhibits cancer initiation [103, 121-123]. These molecular mechanisms during pregnancy may remove or modify more highly susceptible cell types and structures, accounting for the decreased risk of breast cancer [131, 132].

During breast tissue remodeling throughout pregnancy, lobular structures progress to a more differentiated state that makes up the fully developed gland [100]. The undifferentiated Lobules type 1 (Lob 1) structures are most susceptible to carcinogen [133]. In nulliparous women, the breast has the highest portion of Lob 1 structures, with slightly more differentiated Lob 2 present in modest numbers [100, 121]. Throughout breast development during pregnancy, Lob 1 and 2 progress to Lob 3 in the early stages, then to the fully differentiated Lob 4 at the later stages of pregnancy [100, 121]. During involution,

Lob 4 regresses back to Lob 3 [100]. This remains the predominant structure in the once again quiescent gland, which are absent in nulliparous women [100, 121]. Even after full breast development during the reproductive cycle, the mammary epithelium remains responsive to proliferative signaling. However, these stimulated cells have undergone differentiation and can more efficiently metabolize carcinogens and direct a DNA repair response, making them less susceptible to carcinogenesis [100]. Although more differentiated lobular structures are still affected by carcinogen, the malignancy of initiated lesions is decreased with greater degrees of differentiation [133]. Lob 1 gives rise to ductal carcinomas which comprise the majority of breast cancer cases, Lob 2 develops into lobular carcinomas *in situ*, Lob 3 advances to benign breast lesions, such as hyperplastic lobules, cysts, fibroadenomas, and adenomas; and Lob 4 originates lactating adenomas [100, 101, 133]. Thus, differentiation during pregnancy gives rise to different types of lesions and altered susceptibility to breast cancer [100, 101, 122].

Breast cancer susceptibility timepoints due to tissue remodeling:

Post-lactational involution is characterized by extensive tissue remodeling that eliminates a large portion of the mammary epithelium through apoptosis [100]. No other processes or pathological conditions compare to the rapid nature of involution [134]. This reconstruction process is similar to wound healing as the gland is filled with activated fibroblasts and immune infiltration, and the extracellular matrix becomes enriched with high levels of fibrillar collagen, laminin, fibronectin and matrix metalloproteinase activity [135-140]. In fact, the transcriptome of the mammary gland undergoing involution indicates that the same tissue-remodeling processes involved in wound healing and

inflammation are activated during this period, though involution itself is controlled and developmentally programmed [135, 141, 142]. Inflammation is a pro-oncogenic process, such that cancers with an inflammation component have a poorer prognosis than those cancers that do not [143-145]. Due to a microenvironment that mimics wound healing and inflammation, it is believed that involution creates tumor-promoting activities that may support transformation of epithelial cells to cancer cells and allow for tumor cell dissemination [146-148]. This idea has been investigated using rat mammary gland extracellular matrix (ECM) isolated from quiescent nulliparous vs weaning-induced involuting mammary glands. Involution ECM increased breast cancer cell invasiveness and motility as compared to quiescent ECM *in vitro* [139, 145, 147, 149]. A xenograft model of human breast cancer found that rates of metastasis significantly increased when breast cancer cells were mixed with involution ECM compared with cells mixed with nulliparous ECM or Matrigel [147, 148]. Collectively, these studies indicate that involution indeed creates a microenvironment permissive to tumor initiation and represents a metastatic window of susceptibility [139, 147].

A transient increase in breast cancer risk following pregnancy has been established, referred to as pregnancy-associated breast cancer [148]. This cancer is defined as breast cancer occurring within 5 years of pregnancy, and is associated with poor prognosis, an increased rate of metastasis, and a high mortality rate [150-152]. This may appear contradictory to the established protective effect of pregnancy. It is true that an early first full-term pregnancy and multiple pregnancies are associated with a reduction in breast cancer risk for the lifetime of these women [153]. However, a first full-term pregnancy in older women is associated with a perpetual increase in breast cancer risk, possibly due to

a longer period of exposures in undifferentiated breast tissue to estradiol and progesterone prior to pregnancy [153-155]. It is likely that there is a period of tumor-promoting activity during pregnancy due to the growth and invasion-promoting effects of gestational hormones and following pregnancy due to the tumorigenic microenvironment during involution [148, 156, 157]. These risk-associated timepoints would precede the eventual protective effect of pregnancy [120, 153]. Thus, the reproductive cycle both suppresses and promotes breast cancer development [148].

Rat Model of Human Breast Cancer

A comparative genomics approach using a rat model presents an unbiased method of genome-wide screening to identify novel homologous human candidate breast cancer genes that may have been unknown or unsuspected through population studies [158, 159]. These rodent models have the power to identify low penetrance genes or loci that modulate breast cancer risk and translate these to human populations for comparative evaluation [159]. The rat model also allows for exploration of the mechanism through which genes and genetic variants modulate breast cancer susceptibility by studying the natural initiation and progression of mammary cancer. Rat mammary carcinogenesis is a good model of human breast cancer, as induced rat mammary carcinomas display the same histopathological stages and features of malignant breast carcinomas, including epithelial ductal cell origin, progression, and frequency of hormone responsive and non-responsiveness [76, 101, 160-164].

Structure of the rat mammary gland:

The rat has six pairs of mammary glands that are situated along the ventrolateral sides of the body, extending from the cervical glands at the anterior, two pairs of thoracic glands, a pair of abdominal glands, and 2 pairs of inguinal glands at the posterior [165]. These mammary glands appear as subcutaneous sheets of tissue that lay between the skin and body cavity [114]. Mammary tissue is composed of adipocytes and fibroblasts that make up the mammary fat pad, with epithelial structures embedded throughout [166]. The mammary gland can thus be divided into two compartments; the epithelial and the stromal compartments [114]. Adipocytes make up much of the stroma, which is comprised of other cell types, including fibroblasts present within connective tissue and infiltrating vascular endothelial cells and lymphocytes [94, 114]. The epithelium consists of a primary collecting duct that extends from the nipple, with branching luminal ducts whose tertiary branching elongates into smaller ductules [114]. The ductules end as either proliferative terminal end buds, blunt end buds, alveolar buds, or alveoli, with these alveoli developing into milk secreting lobules during lactation [114, 166]. These various epithelial structures all possess distinct morphologies and functionality.

The two main cell types of the mammary epithelium are luminal epithelial cells and myoepithelial cells, which together form bi-layered tubular branching ducts [166-168]. The luminal epithelial cells make up a single cuboidal inner cell layer of ductal structures and develop into the milk-secreting cells of lobuloalveolar structures during lactation [114, 166, 168]. The outer layer is comprised of myoepithelial cells, which secrete a continuous basement membrane that separates the mammary epithelium from the surrounding stroma [114, 117]. Myoepithelial cells contain numerous fine filaments, similar to smooth muscle

cells, which provide a contractile mechanism in response to oxytocin released during lactation [167]. These two cell types work in conjunction during lactation when the outer myoepithelial layer contracts, which squeezes and transports milk released from the inner luminal epithelial layer [94, 167]. Luminal epithelial cells are most relevant to cancer as their cell phenotype is the most similar to breast cancer cells and are thought to give rise to the majority of female breast cancers [114, 117]. Myoepithelial cells are also important in cancer, as their absence within a ductal lesion indicates development into an invasive breast carcinoma [114, 169].

Rat mammary epithelium morphological changes throughout tissue development:

The mammary gland is one of the few organs to undergo most development postnatally, where a large portion of the epithelial ductal structures appear during adolescent rather than fetal development [167, 170, 171]. During fetal growth, the mammary gland develops only as a rudimentary ductal system, comprised mainly of an embryonic mammary stalk that penetrates what is to become the mammary fat pad and with a limited number of branching ducts [170, 171]. At birth, mammary glands grow isometrically in keeping up with normal body growth [170]. It is not until the onset of puberty when hormone-dependent expansive epithelial proliferation occurs and fills the fat pad [170]. This period of development is defined by enrichment of mammary stem cells, high levels of proliferation, epithelial invasion of the mammary fat pad, and ductal morphogenesis [114, 172-174] which is driven by terminal end buds (TEBs) [117]. TEBs are clusters of undifferentiated epithelial cells at the distal end of primary ductules, which form highly proliferative bulbous structures that penetrate the fat pad and result in ductal

elongation and branching [97, 104, 114, 117, 170, 175]. TEBs continuously expand with each estrous cycle, resulting in new primary ducts with secondary and tertiary branches sprouting laterally until this ductal tree has expanded to occupy up to 60% of the mammary fat pad [114, 117, 167, 170, 176]. Alveolar buds develop at the ends of tertiary branching, capable of developing into the lobuloalveolar structures responsible for milk secretion during lactation [117, 167, 170, 176]. Overall, puberty is a critical period of epithelial growth that establishes the ductal tree and primitive lobular structures throughout the mammary fat pad that make up the inactive mammary gland.

The next developmental stage is during pregnancy, when the mammary gland attains maximum development. Pregnancy is comprised of an early phase of growth and a late phase of lobular differentiation [133]. During pregnancy, there is yet a further increase in ductal side branching, which provides connections for the greater number of alveolar buds that develop [94, 114, 133]. These alveolar buds grow and differentiate into functional alveoli, which cluster around terminal ducts to become milk-secreting lobules during lactation [94, 133, 171]. By day 14 of the 21 day gestation period in the rat, there is a distinct increase in both number and size of alveolar lobules [114]. As this intensive ductal side branching and lobuloalveolar formation takes place, there is a continuous increase in the mammary gland epithelium [114, 117, 177, 178]. By late pregnancy, interductal adipose tissue reaches a minimum as the alveolar epithelium comprises most of the mammary fat pad [94, 117]. The expansive proliferation of the alveolar epithelium causes the outer myoepithelial layer to become discontinuous so that the alveolar luminal epithelium comes into direct contact with the basement membrane [114, 168]. This contact does not occur in the ductal epithelium which retains a continuous myoepithelial layer,

accounting for the differences in secretory potential of the ducts vs lobuloalveolar structures [114]. As pregnancy approaches term and growth reaches a maximum, lobules can begin to show some secretory activity prior to their function in lactation [94].

The lactating mammary gland has a distinct appearance from the mammary gland during late pregnancy. Though the mammary gland epithelium continues to display a predominantly lobuloalveolar morphology with fewer ducts, there is a large increase in total cell number which reaches a peak in the first few days of lactation [114, 117, 171, 179]. The lobuloalveolar epithelium also swells osmotically due to increased intracellular lipid and lactose content, causing the outer myoepithelial cell layer to develop an even more discontinuous appearance [114, 168]. During lactation, the luminal epithelium of lobuloalveolar structures function in milk production while luminal epithelium of ducts serves as a conduit for the movement of milk [114, 171]. The myoepithelial cell layer contracts upon stimulation by oxytocin, causing the secretion of milk into the alveolar lumen and the subsequent emptying into the ductules [114, 117]. This lactation cycle persists for about 21 days postpartum, with natural involution beginning in an asynchronous manner near the end of this cycle as pups begin eating other foods [94, 114, 180].

Involution is characterized by remodeling of the mammary gland back to a quiescent state, which is attained by apoptotic activity that decreases the size and number of alveolar lobules whose secretory function is no longer needed [117, 127, 170, 181-184]. Loss of suckling through weaning causes milk to stagnate in the mammary epithelium, initiating the process of involution [94, 183, 185, 186]. There are two phases of involution that have been described. The first phase is a reversible period of involution, where a

resumption in suckling can reestablish a lactation function [94]. During this stage, alveolar cells begin to detach from the basement membrane and undergo apoptosis, resulting in an accumulation of cell debris in the alveolar lumen and lobuloalveolar engorgement due to milk stagnation [94, 114, 168, 186]. The mammary gland enters the second irreversible phase of involution after 2 days of sustained milk stasis and it is only in this stage that tissue remodeling takes place [94, 113, 186]. Proteinases disrupt epithelial junctions and degrade the epithelial basement membrane and extracellular matrix, causing alveoli to regress in size and the myoepithelial cells to attain a more continuous layer [94, 114, 168, 170, 186]. Continued apoptosis results in massive degeneration of the lobuloalveolar structures that once predominated the mammary gland [113, 133, 170, 186]. By six days, most of the secretory lobuloalveolar epithelium is removed and replaced by adipocytes and connective tissue [94]. Ductal structures are regenerated by terminal tubules that continue to branch and reestablish the simple epithelial ductal tree characteristic of the quiescent gland [94, 133].

Figure 2 provides a schematic depicting the gross morphological mammary epithelial changes that occur throughout these key stages of mammary gland development.

Rat mammary epithelial cell proliferation in relation to the estrus cycle:

The onset of puberty in the female rat begins at approximately 30 days of age with the release of the pituitary luteinizing hormone, which stimulates ovarian maturation during this anestrus period [187, 188]. Estrus cycling begins 8-9 days later [189] and occurs every 4-5 days [190, 191]. The human menstrual cycle has an estrogen-dominated follicular stage, a progesterone-dominated luteal stage in which mammary epithelial cell

Figure 2: Mammary gland epithelial changes schematic: (A) Embryonic development - the rudimentary mammary gland begins as a bud containing a limited number of branching ducts. (B) Neonatal development - allometric growth with some extensions to the ductal system. (C) Puberty - epithelial proliferation and invasion of the mammary fat pad establishes the ductal tree. Distal terminal end buds drive ductal elongation and branching. (D) Pregnancy - predominantly lobuloalveolar morphology due to extensive alveolar bud differentiation into milk-secreting lobules. (E) Lactation - the lobuloalveolar epithelium reaches a maximum. The remaining ducts serve as conduits for milk movement. (F) Involution - degeneration of the lobuloalveolar structures and re-establishment of the ductal tree, returning the mammary gland to a quiescent state and the epithelium again predominantly ductal.

(MEC) proliferation reaches a maximum, and a menstrual stage that is characterized by low ovarian hormone levels and MEC apoptosis, balancing cyclical proliferation [192-196]. Similar events occur in the rat estrus cycle, but it is divided into 4 or 5 stages depending on individual classifications: proestrus, estrus, metestrus, which can be further divided into metestrus I and metestrus II, and diestrus [197, 198]. The proestrus phase is a period of high estrogen levels, follicular development, and endometrial proliferation, comparable to the human follicular phase [199, 200]. The estrus phase encompasses ovulation and is marked by increased levels of progesterone, similar to the luteal phase in human menstruation [199]. Early metestrus mimics the human luteal phase and is a progesterone-dominated phase with maximum development of the endometrium [201, 202]. During late metestrus, ovarian hormone levels begin to fall and there is a regression of the endometrium, equivalent to entry into menstruation in humans [199]. Diestrus is characterized by minimal hormone levels, similar to the human menstrual phase, with a beginning rise in estrogen levels towards the end initiating a new round of the estrus cycle [200].

Of the few studies that have analyzed MEC proliferation in rat mammary glands based on estrus cycle staging, there have been conflicting reports, with some indicating peak MEC proliferation during proestrus and diestrus [196, 203], and another study reporting high proliferation during metestrus and early diestrus [199]. Thus, the impact of the estrus cycle on mammary gland biology is still not well characterized. This leaves a necessity to understand the factors involved in normal cyclical growth and regression in the mammary gland as they can be potentially dysregulated during carcinogenesis.

Chemical induction of rat mammary carcinogenesis:

Mammary tumors develop in rats both spontaneously and by oncogene- and chemical-induction. Spontaneous mammary tumors occur frequently in some rat strains and are the most common tumor type in rats [204-206]. Benign mammary tumors appear more often than malignant tumors, and arise mostly from fibrous and connective mammary tissue [206]. Of malignant tumors, fibrosarcomas originating from proliferating stromal fibroblasts are the most common, with one report finding an incidence of 59% of all spontaneous malignant tumors [206]. Mammary tumors are also chemically induced in a reproducibly high percentage of rats, which allowed early researchers to investigate types of carcinogen, routes of administration, and age of recipient rats on the incidence of mammary tumors. Unlike spontaneous tumors, induced tumors are generally carcinomas [206]. Induced rat carcinomas are highly hormone responsive, have short latency, and rarely metastasize; and, since their discovery, have been utilized to simulate human breast carcinogenesis [207-209].

The chemical induction of cancer in rodents was first observed by Maisen and Coolen who applied the carcinogen 3-methylcholanthrene (3-MC) onto the skin of mice [210]. Later it was found that the feeding of polycyclic hydrocarbons induced mammary cancer preferentially in rats [211, 212]. The first compound used in this way was 2-acetylaminofluorene (2-AAF), which was incorporated into the food of rats over several months [213]. Building on these studies, it was discovered that up to three IV injections of polycyclic aromatic hydrocarbons such as 7,12-dimethylbenz(a)anthracene (DMBA) resulted in a high incidence of female mammary cancer in 80% of the Wistar and Sprague-Dawley strains of rats [214, 215]. However, *Huggins et. al* was the first to show a single

oral dose of DMBA administered to 50-65 day old female Sprague-Dawley rats resulted in 100% mammary carcinoma incidence in just 2 months, and was much more effective than similar administration of 3-MC and 2-AAF that required several doses [212]. A positive correlation was found between dose of carcinogen and incidence of mammary tumors. An optimal oral DMBA dose of 20 mg/kg body mass in sesame oil was published and is now a standard procedure [212, 216]. The DMBA-induced rat mammary tumor model became known as the “Huggins tumor” and is the most widely utilized animal model of human breast cancer [217]. This discovery of a single dose of carcinogen was crucial as it allowed for the study of cancer initiation, carcinogenesis, and tumor biology, while providing the most adequate experimental model to study breast cancer development [217].

DMBA model of rat mammary carcinogenesis:

The DMBA-induced rat mammary tumor model is the most efficient method to study human breast cancer as tumors develop quickly and in the form of invasive ductal carcinomas, which account for 80% of diagnosed breast cancers worldwide [218]. DMBA-induced mammary tumors can be reproducibly induced at a high frequency and develop on average 5 weeks following administration [211, 219]. These mammary tumors are multifocal and occur at different sizes within the same animal, eventually resulting in skin ulceration, necrosis, infection, and death [219]. DMBA-induced mammary cancer displays similar pathology to human breast cancer, including a common site of disease initiation and morphology throughout tumor progression [219-221]. The carcinogenic activity of DMBA is induced by metabolic activation [222]. The liver is the major site of metabolic activation for many chemical carcinogens, but the highly lipophilic nature of polycyclic

aromatic hydrocarbons, including DMBA, results in accumulation in fatty tissue [222, 223]. DMBA concentrates in the significant amount of adipose tissue that comprises the mammary gland and local metabolic activation occurs [223].

Age is a critical factor in the DMBA mammary carcinogenesis model, as the incidence of tumors reaches a peak when administered to female rats at 50-55 days old, before rapidly declining [175, 224-226]. Tumors will rarely be induced in rats older than 100 days [223]. This 50-55 day timepoint coincides with peak DNA synthesis, as shown through ³H-thymidine incorporation in mammary gland DNA that reaches a maximum at 50 days of age [225, 226]. The terminal ductal structures in mammary glands are believed to be the site of action by DMBA as they are highly proliferative and display an elevated rate of DNA synthesis at 50-55 days of age [217, 227]. DMBA is metabolically activated by CYP1A1 and CYP1B1 isoforms from the P450 family of metabolic enzymes, which creates unstable diol epoxides [228, 229]. These epoxides bind and form adducts onto the DNA of proliferative mammary structures [222, 230]. Notably, DMBA itself is capable of inducing expression of CYP1A1 and CYP1B1 in the mammary gland, which can further increase metabolic activation [222]. DNA adducts of DMBA metabolites hinder DNA repair enzymes and cause mutations, which contributes to malignant transformation of epithelial cells [222, 223, 231]. The intrinsic affinity of DMBA to collect in mammary tissue and the ability of mammary tissue to locally activate DMBA creates a clean, mammary-specific model of carcinogenesis.

Mammary Carcinoma Susceptibility Quantitative Trait Loci

Susceptibility alleles:

Rat strains vary in their susceptibility to carcinogen-induced development of mammary cancer [232]. The inbred Copenhagen (COP) strain displays nearly complete resistance to mammary carcinogenesis, developing an average of 0.25 carcinomas per rat with a single dose of DMBA [159, 164, 232, 233]. In contrast, the highly susceptible Wistar Furth (WF) strain displays >90% tumor incidence after a single dose of DMBA [159, 160, 164, 232, 233]. These divergent mammary cancer susceptibility phenotypes between strains allow for the ability to isolate genetic loci that contribute to mammary cancer susceptibility [159]. Inbred rat strains that differ in their susceptibility to DMBA-, N-methyl-N-nitrosourea (NMU)-, or estrogen-induced mammary carcinogenesis have been used to predict locations of quantitative trait loci (QTL) that control susceptibility to mammary carcinogenesis, termed the *Mammary carcinoma susceptibility (Mcs)* and *Estrogen-induced mammary cancer (Emca)* loci [164, 234-239]. Quantitative trait linkage analysis studies were conducted in the 1980s using segregating backcrosses between WF and either resistant rat strain COP or Wistar Kyoto. These studies identified eight QTLs that modified susceptibility to DMBA-induced mammary cancer, termed mammary carcinoma susceptibility loci 1-8 (*Mcs1-Mcs8*) [77, 158, 161, 164, 234, 240]. *Mcs1-4* are present in the COP strain genome while *Mcs5-8* stem from the Wistar Kyoto (WKy) genome [164, 241].

Congenic rat lines carrying a specific *Mcs* on a WF genetic background have been used to validate these QTLs [76, 161]. Congenic lines are generated by first mating two inbred rat strains to create an N1 heterozygote generation. In subsequent matings, one strain

is considered the donor (for *Mcs* analysis this is COP or WKy) and the other is the recipient (WF). The N1 generation is backcrossed to the recipient strain for up to ten generations. Within each generation, animals are genotyped at the predicted region and selected for based on possession of the desired donor allele. This results in introgression of the donor allele at the region of interest into a recipient genetic background. The phenotypes of congenic lines are then compared to inbred parental strains to determine if the isolated region of interest modulates the overall phenotype. This breeding scheme is diagrammed in Figure 3. Of the COP loci, it was found that *Mcs4* contributes to increased susceptibility while *Mcs1-3* decrease susceptibility [164]. Even though resistance to mammary carcinomas is a multilocus trait in the COP genome, when rats are homozygous for *Mcs1-3*, this is enough to suppress tumor development regardless of *Mcs4*, indicating these three QTLs account for the majority of the phenotypic differences between WF and COP [164].

To limit the set of candidate genes that confer disease susceptibility, the QTL must be fine mapped to a higher resolution using genetic markers, such as single nucleotide polymorphisms (SNPs) and microsatellites, spaced at regular intervals across the QTL [161, 240]. Congenic lines harboring the *Mcs* chromosomal region are repeatedly backcrossed to the WF susceptible genetic background to produce lines that carry delimited QTL intervals due to homologous recombination [240]. These new lines are phenotyped for retained mammary cancer susceptibility or resistance following DMBA administration [161, 240]. These fine mapping studies in congenic lines allow the smaller selection of potential candidate genes that can be extended to orthologous human studies of breast cancer risk [158, 161]. The work presented in this dissertation will involve two different mammary cancer susceptibility loci, *Mcs3* and *Mcs1b*.

Figure 3. Generation of congenic animals. Adapted from Kim et al. (2010) [242]. Mating of two inbred rat strains generates a heterozygous N1 generation. These animals are backcrossed to the recipient strain and this cycle of backcrossing repeated for 10 generations. At each generation animals are genotyped to ensure the desired donor allele is present in the region of interest. Heterozygotes are crossed to yield offspring homozygous for the donor region of interest. This method introgresses the donor allele (black bar) into the recipient genome (white bars).

Mcs3:

From linkage analysis using the susceptible WF and the resistant COP strain post-DMBA treatment, *Mcs3* was initially predicted to reside within a 30 cM region of rat chromosome one that acted as a dominant resistance loci [164]. The COP resistant *Mcs3* was physically confirmed and delimited to a 29.4 Mb region on rat chromosome one in 2017 and results in around a 50% reduction in mammary cancer susceptibility in homozygous rats [243]. This rat region aligns to four syntenic regions of human chromosomes 11 and 15 and four other regions of the human genome that contain a single gene, with 198 genes in common or orthologous between these species [243]. Most notable in the context of breast cancer, rat *Mcs3* contains a sequence orthologous to human 11q13/14, which is amplified in a subset of female breast carcinomas with poor prognosis [243, 244]. Potential breast cancer risk associated variants at *Mcs3* orthologous loci 2p16.3, 11p15.4, 11q14.1, 11q14.2, and 15q25.2 have also been identified by population-based genetic association studies [243, 245-247]. The *Mcs3* locus is currently too large for functional studies so fine mapping is needed to reduce the pool of candidate genes. Work with *Mcs3* in this dissertation focuses on evaluation of expression of known breast cancer genes in this locus and further fine mapping to increase the resolution at this QTL.

Mcs1b:

Another independently acting Mcs QTL, *Mcs1*, was first identified in 1998 as a susceptibility locus that modulates tumor multiplicity phenotype [164]. Genetic linkage analysis of (WF.COP)F₁ x WF backcrosses with mammary carcinogenesis induction by DMBA predicted *Mcs1* localized to a 30 cM region at the centromeric end of rat

chromosome 2 [164]. Gould and his group created an *Mcs1* congenic line by transferring the *Mcs1* COP allele onto a WF genetic background [159]. In delineating the large *Mcs1* locus, recombinant lines were created by backcrossing the *Mcs1* congenic line to the WF strain. These new congenic lines were phenotyped for their sensitivity to DMBA-induced mammary cancer. COP-homozygous rats from three nonoverlapping lines showed resistance to DMBA-induced mammary tumorigenesis. From these lines, Gould's group identified three *Mcs1* subloci independently capable of conferring mammary cancer resistance, termed *Mcs1a*, *Mcs1b*, and *Mcs1c*. This positional mapping of *Mcs1* determined that this locus contains more than one gene that contributes to mammary cancer susceptibility.

Mcs1b, was mapped to a region of 13 cM by Gould's group, which resulted in a 58% reduction in DMBA-induced mammary carcinomas per rat as compared to the WF strain [159]. In 2012, *Mcs1b* was further fine mapped and delimited to a 1.8 Mb region corresponding to rat Chr2:42364155-44195382 (Rnor_6.0) [248]. Females homozygous for this delimited COP allele displayed 56% fewer mammary carcinomas compared to susceptible WF females [248]. The current *Mcs1b* is highly relevant to human breast cancer as it contains a genomic sequence orthologous to human 5q11.2, a breast cancer susceptibility locus identified in multiple GWA studies [245, 248-252]. Genetic variation at 5q11.2 susceptibility allele is associated with breast cancer invasive ductal carcinoma, invasive lobular breast carcinoma, ER+, ER-, and triple negative and breast cancer subtypes [253-255].

Mapping of *Mcs1b* to a much shorter genomic interval allows for selection of candidate breast cancer susceptibility genes and functional studies of this QTL. Within the

overlap of rat *Mcs1b* and orthologous human 5q11.2 are two genes, *mitogen-activated protein kinase kinase kinase 1* (*MAP3K1/Map3k1*) and *MIER family member 3* (*MIER3/Mier3*). Present in *Mcs1b* but not 5q11.2 is *GC-rich promoter binding protein 1* (*Gpbp1*) (Figure 4). Rat *Mcs1b* was found not to contain any protein-coding differences between COP and WF alleles, suggesting that differences in mammary cancer susceptibility may be due to variation in one or more nonprotein-coding regulatory elements that differentially control gene expression between *Mcs1b* genotypes [248]. Mammary gland and mammary carcinoma transcript levels of *Mcs1b* genes were measured in both DMBA-exposed and age matched control WF and *Mcs1b* females. The effect of mammary cancer susceptible and *Mcs1b* resistant genotypes were statistically significant for *Gpbp1*, *Mier3*, *Map3k1*. However, only *Mier3* showed significant expression differences when comparing *Mcs1b* genotypes of females exposed to DMBA [248]. Additionally, there were no significant *Mier3* transcript differences between *Mcs1b* resistant and susceptible genotypes in spleen, thymus, ovary, or brain tissues, indicating *Mier3* differences between *Mcs1b* alleles may be specific to mammary gland tissue [248]. Work with *Mcs1b* in this dissertation focuses on further expression analysis of *Mier3* within rat mammary glands through mammary cancer progression and time windows of susceptibility. Additionally, I investigate the molecular function of MIER3 in human breast cancer through identification of protein-protein interactions.

The MIER family:

The MIER gene family consists of three related genes that were named based on shared homology between their ELM2 and SANT domains. The prototypical member,

Figure 4. Rat *Mcs1b* and orthologous 5q11.2 breast cancer risk allele share two human/rat conserved transcripts. Adapted from denDekker et al. (2012) [256]. Genetic markers used to map *Mcs1b* to this region are marked on the X-axis. The ends of congenic lines used to delimit *Mcs1b* are included for orientation (white is susceptible, gray is resistant). Potential candidate breast cancer susceptibility genes *Map3k1* and *Mier3* overlapping *Mcs1b* and 5q11.2 are shown as filled black bars that represent exonic and intronic DNA.

MIER1, was first discovered as a fibroblast growth factor early response gene [257]. Hence, the original nomenclature Mesoderm Induction Early Response (MIER) genes. Human MIER1 is a single copy gene on chromosome 1 that consists of 17 exons and gives rise to four major protein isoforms due to alternative splicing and promoter usage but retain a common internal region with functional domains [258, 259]. There are two C-termini variants named MIER1 α and β , with β arising from the inclusion of a facultative intron [260]. The α C-terminus contains 23 amino acids and includes an LXXLL motif, canonically responsible for interaction with nuclear hormone receptors [259, 261, 262]. The β C-terminus contains 102 amino acids and possesses a functional nuclear localization signal [259, 263]. To date, much of the research into MIER1 has focused on MIER1 α and β , both of which have been shown to function as transcriptional repressors [261]. There are also two major MIER1 isoforms that differ at their N-termini as a consequence of alternate promoter usage, named P1 and P2 [259]. MIER1 is transcribed from P2, and MIER-3A is transcribed from P1 and includes an additional exon (3A) [261, 262]. This encodes 25 amino acids that contain a nuclear export signal [259, 261, 263]. MIER2 is a single copy gene on chromosome 19 consisting of 14 exons that is transcribed into a single transcript. It also possesses internal ELM2 and SANT domains, as well as a predicted nuclear localization signal. MIER3 is a single copy gene on chromosome 5 consisting of 13 exons and giving rise to 5 isoforms due to alternative splicing. Isoform 1 is the canonical sequence and isoform 3 only differs by a single amino acid deletion in the ELM2 domain. There is a 5 amino acid insert near the N-terminus of isoform 2, and isoform 4 is missing the first 63 amino acids. These isoforms possess the ELM2 and SANT domains common to this gene family, as well as a predicted nuclear localization signal. MIER3 also has two LXXLL

sequence motifs, one upstream of the ELM2-SANT domains and another within the ELM2 domain (Figure 5). Isoform 5 is a truncated variant of only 119 amino acids at the N-terminus and lacks the ELM2 and SANT domains [264]. Human MIER1 and MIER3 are similar, with BLAST analysis showing 54% identical amino acid sequence [265]. However, MIER3 differs in its possession of two LXXLL sequence motifs as compared to one within MIER1. Co-immunoprecipitation analysis has also demonstrated that the MIER family members do not dimerize and act autonomously from each other [266]. Thus, although similar in sequence and protein domains, the MIER family may possess differing functional roles.

Within the MIER family, a functional role of MIER1 in transcriptional regulation has been well characterized, though there is little published research functionally annotating MIER2 and MIER3 [266]. Co-immunoprecipitation and mass spectrometry-proteomic approaches have identified the ELM2 domain of MIER1 α and MIER1 β as necessary to recruit histone deacetylase 1 and 2 (HDAC1/2) and repress transcription through histone remodeling complexes [263, 266-269]. In addition to HDAC recruitment, MIER1 has other mechanisms of transcriptional repression, including physical association with the histone acetyltransferase CREB-binding protein (CBP) and inhibition of its activity [270]. The SANT domain of MIER1 can also disrupt promoter binding by the transcription factor Sp1 and alter downstream transcription of target genes [263, 266].

Proteomic studies of Bromo-Adjacent-Homology domain-containing 1 (BAHD1) have reproducibly co-purified this protein with each of the MIER proteins and HDAC1/2 [271]. BAHD1 acts as a transcriptional co-repressor by interaction with other proteins to

MIER3 (NP_001284527.1)

MAEASFGSSSPVGSLSSEHDHFDPTAEMLVHDYDDERTLEEEEMMDEGKNFSSEIEDLEK
EGTMPLEDLLAFYGYEPTIPAVANSSANSSPSELADELPDMTLDKEEIAKDLLSGDDEET
QSSADDLTPSVTSHETSDFFPRPLRSNTACDGDKESEVEDVETDSGNSPEDLRKEIMIGL
QYQAEIPPYLGEYDGNEKVYENEDQLLWCPDVVLESKVKEYLVETSLRTGSEKIMDRISA
GTHTRDNEQALYELLKCNHNIKEAIERYCCNGKASQEGMTAWTEEECRSFEHALMLFGKD
FHLIQKNKVRTRTVAECVAFYYMWKKSERDYDFAQQTRFGKKRYNHHPGVTDYMDRLVDE
TEALGGTVNASALTSNRPEPIPDQQLNILNSFTASDLTALTNVATVCDPTDVNCLDDSF
PPLGNTPRGQVNHVPVVTEELLTLPSNGESDCFNLFETGFYHSELNPMNMCSEESERPAK
RLKMGIAVPESFMNEVSVNNLGVDNFENHTHHITSAKMAVSVADFGSLSANETNGFISAH
LHQHAALHSE

LXXLL Motifs

ELM2 Domain

SANT Domain

Nuclear localization signal

Figure 5: Protein domains of MIER family member 3 (MIER3). MIER3 possesses a juxtaposed ELM2 and SANT domain. One LXXLL sequence motif is near the N-terminus and the other is within the C-terminal region of the ELM2 domain. There is also a nuclear localization signal near the C-terminus of the protein.

promote chromatin condensation [271]. As such, BAHD1 is reported to consistently interact with the chromatin writer G9a, readers HP1 $\alpha/\beta/\gamma$, CDYL1/2, MBD1, and remodelers CHD3, RUVBL1/2 [271]. These data provide evidence for the first time of the other MIER proteins as subunits of a multiprotein complex that may have a role in transcriptional repression.

MIER1 function in breast cancer:

An immunohistochemical study of the MIER1 α protein expression pattern in human tissues found expression in all endocrine and reproductive tissues except the thyroid gland, with the majority of non-endocrine tissues negative for MIER1 α [259]. The most intense staining was found in the ovaries, testis, breast, pancreas, and adrenal gland and pituitary. The subcellular localization of MIER1 α was cytoplasmic across all tissues examined except oocytes, germinal epithelial cells, and ductal epithelial cells of the breast which were exclusively nuclear [259]. This is concordant with findings in mice, in which there was no immunoreactivity of MIER1 α in most tissues, except for high levels in endocrine tissue, and localization was largely cytoplasmic [272]. These collectively suggest an endocrine role of MIER1 α . In the breast specifically, another study found very low *MIER1* mRNA levels in non-diseased breast cell lines and tissue, but significantly higher expression in breast cancer cell lines and tumor tissue, highlighting an association with human breast cancer [273]. It has been reported that there is no difference in MIER1 α protein levels between non-diseased breast tissue and breast carcinoma tissue, but there is a drastic shift from nuclear to cytoplasmic subcellular localization with progression to invasive ductal carcinoma [274]. Nuclear MIER1 α was detectable in 75% of normal tissue,

51% of DCIS and only 4% of IDC [274]. It has been demonstrated that interaction of HDAC1 and 2 are necessary for the nuclear translocation of MIER1 α , and that insulin or insulin-like growth factor induces cytoplasmic localization in breast cancer cells [262, 275]. Additionally, inclusion of exon 3A in the MIER1-3A α isoform results in cytoplasmic translocation due to inclusion of a nuclear export signal but has no effect on MIER1-3A β localization [261]. MIER1 α can also physically bind estrogen receptor alpha (ER α) with and without the presence of estrogen, and it has been shown that overexpression of MIER1 α inhibits estrogen-stimulated growth of breast cancer cells [274]. These results indicate that a combination of alternative splicing and interacting partners effects the subcellular location of MIER1 α in the context of breast cancer, and the potential function of MIER1 α as an ER α corepressor could either indicate an additional non-genomic role of MIER1 or enhance ER α non-genomic activities in breast cancer development.

MIER3 function in breast cancer:

Human MIER3 localizes to the nucleus of breast cancer cells and *MIER3* levels are elevated in invasive ductal and invasive lobular carcinoma samples compared to normal breast tissue [256]. However, much of what is known about MIER3 comes from studies of the rat ortholog. Human and rat MIER3/Mier3 (GenBank ref NP_689835.3 and NP_001161472.1) gene products share 93% amino acid sequence identity. While 5 isoforms have been isolated in the human, there are 3 reported Mier3 isoforms in the rat (NCBI). One isoform differs from the full-length transcript via intra-exonal splicing of a 363 bp segment in exon 1, resulting in an alternate translational start site at position 1013 and a 23 amino acid truncation of the N-terminus. The other isoform utilizes an alternative

transcription start site upstream of the normal start site, which results in usage of three short exons in place of a skipped exon 1 and slightly alters the N-terminus. These result in full-length, spliced, and alternative 5'-transcript start site variants of *Mier3*. Rat *Mier3* is contained within the mammary cancer susceptibility locus *Mcs1b*. Using rat lines that are resistant or susceptible to mammary cancer based on inclusion of this locus, lower levels of the alternative start site variant (*Mier3-alt5'P*) has been found in the resistant strain. However, no differences were identified between the full-length and splice variant forms (Samuelson Lab, unpublished). This indicates that lower levels of *Mier3* are genetically determined and transcript variant-specific, and that while loss of MIER1 may contribute to breast cancer progression, a reduction in *Mier3* expression appears to be protective against mammary carcinoma development. Additionally, no differences in *Mier3* expression were found between rat strains in spleen, thymus, or ovary tissue (Samuelson Lab, unpublished), further indicating *Mier3* expression differences and downstream effects on cancer development occurs in a mammary gland autonomous manner.

Dissertation Overview

Overall goal:

This dissertation focuses on using WF.COP *Mcs3* congenic lines to fine map this locus with the goal to narrow the genetic interval and reduce the pool of candidate susceptibility genes for comparative analysis in human breast cancer risk. In addition, analyzing *Mier3* expression and protein function in this dissertation begins to identify the role of MIER3 in disease progression and developmental timepoints of altered

susceptibility. The goal here is to identify a MIER3-driven mechanism of breast cancer susceptibility for future preventative or screening strategies.

Hypothesis and Aims:

The hypothesis is that (1) the *Mcs3* locus contains genetic elements that control mammary cancer susceptibility, and (2) MIER3/Mier3 has a functional role in human breast cancer/rat mammary cancer development.

Aim 1: Physically narrow *mammary carcinoma susceptibility 3* (*Mcs3*) region on *RN01* using WF.COP congenic rat lines.

Aim 2: Associate *Mier3* expression with mammary gland cell type, mammary cancer progression, and time windows of susceptibility

Aim 3: Identify protein interactions of MIER3 (specifically determine if MIER3 interacts with nuclear hormone receptors)

CHAPTER II

CHEMICAL CARCINOGEN INDUCED RAT MAMMARY CARCINOGENESIS IS A POTENTIAL MODEL OF P21-ACTIVATED KINASE POSITIVE FEMALE BREAST CANCER

Introduction:

Amplification of human chromosome *11q13/14* in breast cancer is associated with poor prognosis, with patient tumors displaying chemoresistance and a 10 year survival rate of only 50% [244, 277-280]. Amplification of *11q13/14* contains several known and putative driver genes, but it remains unclear if a cancer phenotype is driven by a cassette of genes, or a single oncogene [279, 281]. A recent study which profiled nearly 2500 primary breast tumors with copy number aberrations determined *11q13/14* contains two gene cassettes, one centered around cyclin D1 (CCND1, 11q13.3), and the other around p21 activated kinase (PAK1, 11q14.1) [278].

Human breast epithelial cells normally have low *PAK1* expression; however, amplification of PAK1 is found in 33% of breast tumors and cancer cell lines [282-285]. *PAK1* gene expression is associated with aggressive breast cancer type, histologic grade and proliferation level [283, 285, 286]. A meta-analysis of 15 independent studies that

Adapted from Duderstadt et al. (2021) [276]. See Appendix

investigated association of PAK1 with disease prognosis, found high *PAK1* expression in tumors is negatively associated with overall survival and disease-specific survival [287]. Expression of PAK1 is also relevant to breast cancer survival, as its expression inhibits antiestrogenic action of tamoxifen in tamoxifen-sensitive breast cancer cell lines. Furthermore, *PAK1* expression is linked to tamoxifen resistance and disease recurrence in primary breast carcinoma samples [283, 288].

The *PAK1* gene product is a serine/threonine kinase involved in regulating cell morphology and motility through mediation of actin organization [289]. *In vitro* expression of PAK1 is implicated in a broad array of signaling processes that correspond to breast cancer-related phenotypes, including cell migration, invasion, and anchorage-independent growth [290, 291]. Regulation of anchorage-independent growth signaling and development of invasive breast carcinomas is mediated by proteins that directly interact with PAK1, including cyclin-D1 (CCND1), mitogen-activated protein kinase-1 (MAPK1), and RAS-homolog-family-member-A (RHOA) [282, 292]. Overexpression of PAK1 has been shown to lead to focal adhesion complex disassembly, formation of lamellipodia and filopodia, as well as colocalization with actin filaments at the leading periphery of cells [289, 291, 293].

The *Mammary carcinoma susceptibility (Mcs3)* QTL, a 27.8 Mb segment on rat chromosome 1 that controls susceptibility to developing mammary carcinomas, contains sequence orthologous to human *11q13/14* [243]. These orthologous sequences contain rat/human *Pak1/PAK1* orthologs. *Integrin-linked kinase (ILK)*, another *Mcs3*-nominated candidate gene, [243] is on a different human chromosome than PAK1; however, a physical interaction between ILK and PAK1 has been documented [294]. Similar to *PAK1*,

expression of *ILK* is associated with breast cancer and five-year survival [295]. *PAK1* and *ILK* expression are both associated with female breast cancer [282, 290, 295], and rat orthologs of these genes are located at *Mcs3*. We sought to determine if expression of either rat *Pak1* or *Ilk* is associated with rat *Mcs3* genotype and DMBA-induced rat mammary carcinogenesis, using our integrated model of breast cancer susceptibility and development [163, 296].

Methods:

Congenic Strains and Animal Breeding

Wistar Furth.Copenhagen (WF.COP) congenic rat strains D and E used in this study are described in [243]. Briefly, strain D contains a segment of Copenhagen (COP/NHsd) *RNO1* from markers *DIRat277* (Chr1:134,120,332-134,120,480) to *DIRat321* (Chr1:171,716,519-171,716,714). Strain D is positive for the *Mcs3* COP allele associated with decreased mammary cancer susceptibility. Strain E contains a segment of Copenhagen (COP/NHsd) *RNO1* from markers *DIRat214* (Chr1:105,499,947-105,500,046) to *DIRat350* (Chr1:141,580,935-141,581,089). Strain E is negative for an independently acting *Mcs3* decreased susceptibility allele. Rat chromosome physical positions are from UCSC rat genome assembly Rnor_6.0 [297, 298]. Animals were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care approved facility on a 12-hr light/dark cycle. LabDiet 5001 Rodent Diet (PMI Nutrition International) and acidified water was provided *ad libitum*. All protocols involving animals were approved by the University of Louisville Institutional Animal Care and Use Committee.

DMBA Administration and Tissue Collection

At 50-55 days of age, 7,12-Dimethylbenz(a)anthracene (DMBA Acros Organics, CAS#: 57-97-6, 20 mg/mL in sesame oil) was given by single oral gavage (65 mg DMBA/kg body mass) to 10 WF.COP-RN01 strain D and 11 strain E females to induce mammary carcinogenesis. Age matched females (9 strain D and 12 strain E) not exposed to DMBA were used as untreated controls. Four weeks following DMBA administration, the DMBA-exposed and age-matched non-exposed rats were euthanized by CO₂ asphyxiation. Abdominal-inguinal mammary glands were flash frozen and stored at -80° prior to RNA extraction. Right thoracic mammary glands were formalin fixed and paraffin embedded (FFPE) for use in immunohistochemistry (IHC).

Quantitative PCR

Mammary tissue was homogenized in TRI-reagent (Molecular Research Center TR118) for total RNA extraction. Three molar sodium acetate (1/10 v/v) followed by a 100% ethanol (2.5x v/v) wash was used to remove potential solvent contaminants. Samples were then treated with Turbo DNase (Life Technologies). Reverse transcription reactions, containing 1µg RNA, 0.5x RNasequre, 5µM random hexamers (ThermoFisher N8080127), 25ng/µL oligo dT18 (ThermoFisher 18418012), and 0.5 mM dNTPs (ThermoFisher 10297018), were incubated for 5 minutes at 65° C. First strand buffer (1x), 100mM DTT, and 1µL Superscript IV reverse transcriptase (ThermoFisher 18090010) were added and incubated 5 m at 25° C, 1 h at 50° C, and 15 m at 70° C. Quantitative PCR reactions containing 1x TaqMan buffer A (ThermoFisher), 3.5 mM MgCl₂, 0.2 mM dNTP, 500 nM each forward and reverse primer, 200 nM target TaqMan gene-expression assays

(ThermoFisher) for *Pak1* (Rn00664986_m1), *Ilk* (Rn00591471_m1), or *Rplp2* (Rn01479927_g1), 0.025 U/μl Taq Gold DNA pol (ThermoFisher 18080240), and 50 ng cDNA were run at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. TaqMan gene-expression assay probe and primer sequences were:

Pak1 FAM-ACTATCTGGACAGTTACCTTG with forward 5'-TGGTCATGAGGGAAAACAAAAA and reverse 5'-TCCATGACAACCCATAGCTCATC; *Ilk* FAM-CGGCTCAGGATTTT with forward 5'-GCAGGGACTTCAATGAGGAATG and reverse 5'-CGGAAGCACGTTAGGATGAGA; and *Rplp2* VIC-CTGAATGGAAAGAATATTGAG with forward 5'-TGAACGACTCAACAAGGTCATCA and reverse 5'-CAACACCCTGAGCGATGACA. The manufacturer indicates that amplification efficiency is 100% ± 10% for TaqMan gene-expression assays [299]. Amplification efficiencies for TaqMan assays ranged from 95 to 99% in our experiments. Quantitative PCR was conducted using an ABI Prism 7900HT Sequence Detection System with SDS v2.3 software (Applied Biosystems).

Immunohistochemistry

Tissue was cut in 4 μm sections from FFPE blocks onto superfrost plus microscope slides (VWR). Slides were deparafinized in xylene and ethanol, boiled in sodium citrate buffer (10mM sodium citrate, 0.05% tween, pH 6.0) for 20 minutes, washed in 1X TBS with 0.025% Triton X-100 for 10 minutes, then blocked in 10% normal goat serum with 1% BSA in TBS for 2 hours. Primary antibodies were diluted 1:100 in TBS with 1% BSA and kept with samples overnight at 4°C (Anti-PAK1 Abcam131522, rabbit IgG Abcam

37415). Slides were visualized using an HRP/DAB detection IHC kit (Abcam ab64261), counterstained with hematoxylin, destained using xylene and ethanol, and coverslipped with ProLong Gold antifade mountant (Thermofisher P36930). Slides were imaged at 40x magnification on an Aperio Imagescope 12.4 Software (Leica Biosystems).

De-identified human breast disease clinical samples for IHC were from female patients seen at the UofL James Graham Brown Cancer Center (JG-BCC). Samples were obtained under an approved UofL IRB protocol to protect the privacy of research subjects.

The intensity of DAB staining of IHC samples was quantified using open-source ImageJ Fiji software [300]. Corresponding tissue sections were quantified for Pak1 and control IgG. Microscopic images were submitted to the ImageJ plug-in vector HDAB, which color deconvolutes and outputs separate images of DAB staining and hematoxylin counterstaining. Mammary ductal structures were outlined in the deconvoluted DAB image output and mean intensity measured. The intensity value range is from 0, representing the darkest shade, to 255, representing a pure white, 8-bit image. Intensity values of Pak1 and IgG images were converted to optical density (OD), using the equation:

$$OD = \log (\text{max intensity}/\text{mean intensity})$$

where maximum intensity is 255. Ratios of Pak1 OD to IgG OD for each image were used to determine relative Pak1 staining. Ratios were binned and assigned to a histogram profile using GraphPad Prism version 7.00. A gaussian curve was fit to this plot. Samples with ratios that were greater than one standard deviation from the mean were counted as positive for Pak1 staining.

Sanger Sequencing and Strain Comparison

A reference rat *Pak1* gene sequence was obtained from Ensembl ID ENSRNOG00000029784. Primer3 plus software was used to design PCR primers flanking Pak1 intron/exon splice sites and UTRs based on rat reference sequence RGSC 6.0/rn6. Primers and sequences are located in table 1, which is available at Mendeley Data (<http://dx.doi.org/10.17632/s5ywd3525d.1>) [301]. The UCSC genome browser *in silico* PCR tool was used to exclude off target primers. Genomic DNA was extracted from rat spleen tissue, and PCR amplified at cycling conditions of 94°C for 2 minutes, 18 cycles of 94°C for 15 seconds and 68°C for 2 minutes, 22 cycles of 94°C for 15 seconds and 59°C for 2 minutes, and 59°C for 10 minutes. PCR samples were Sanger sequenced using an ABI PRISM 3130xl Genetic Analyzer. Sequences were compared between WF, COP, and WF.COP-RN01 strain D rats. Identified variants were given dbSNP identifiers from NCBI and submitted to the European Variation Archive (EVA) project and Rat Genome Database ID. The database, UTRdb, contains curated 5' and 3' UTRs of eukaryotic mRNAs [302] and was used to annotate functional UTR elements. The miRNA database miRBase v22 was used to annotate miRNA binding sites within UTRs [303]. When comparing sequences at intron/exon junctions, the consensus 5' splice donor sequence GG(cut)GTRAGT and 3' splice acceptor site CAG(cut)S were used, where R denotes any purine and S represents G or C. Branch sites were searched between 18-40 nucleotides upstream from the 3' end of an intron using the consensus sequence YYRAY, where Y indicates a pyrimidine and R denotes any purine.

Table 1: Rat Pak1 Sequencing Primers

Ensembl ID of target	Lab Name	Forward Primer Sequence	Reverse Primer Sequence	Position (RN01) RGSC 6.0/rn6	Amplicon (bp)
ENSRNOE000000319951	rPak1 exon2	GGGGGAGAGACTGGGTTACT	GTCCTGAGCAACGAGTCAGT	162830750-162831333	584
ENSRNOE000000321430	rPak1 exon3	CCACTCCATACTTTATAGCCTTTTGG	TTAAGAGGCCCATGACAGC	162838721-162839231	511
ENSRNOE000000306605	rPak1 exon4	CCTGCTCTGAAGCGGTAAA	ACTGCTTACCATGGCATGCA	162839289-162839851	563
ENSRNOE000000308069	rPak1 exon5	AGCATGGCTTCTGTCAAGGA	TGTCGGGAAACACATCCAGG	162843790-162844329	540
ENSRNOE000000314791	rPak1 exon6	CAAAGGCCCTTGGTTGATGAGT	GGTGGTTCAGCTGGACATAGT	162857566-162858088	523
ENSRNOE000000305112	rPak1 exon7	GGGCTGTGGCTCAGAAAG	AGCTGGTGAACACAGACAGG	162860337-162860861	525
ENSRNOE000000301435	rPak1 exon8	TGGCTGGTATGCTTTTGGGA	GACAGCAATGCACGTGTGA	162862360-162862957	598
ENSRNOE000000302903	rPak1 exon9	AGGGCTACAGAGGGAGGATG	TCACCCACCTGGAAGTTGTC	162865817-162866340	524
ENSRNOE000000309613	rPak1 exon10	TGCGCAGTAAATGTTGTGGC	ACAACACCCGATGGAAGTGA	162869936-162870437	502
ENSRNOE000000299955	rPak1 exon11	TCTTCAAAGGCTGGCTGAGA	GGAAGCAAGGACAAATGTGTGT	162871624-162872187	564
ENSRNOE000000296239	rPak1 exon12	CCTGGAGGACTTACTTCCTTTGT	CGGAAGAGAGACTGGCGTGTAA	162874910-162875486	577
ENSRNOE000000297756	rPak1 exon13	GCCTGTGCTGCCCATGTGTAA	GGCAGGCTGTGATAACTGGT	162875943-162876497	555
ENSRNOE000000304391	rPak1 exon14	GGCATCAGAGCTGCATCAGA	ACACCCAAACTCTGACAGAGG	162878819-162879353	535
ENSRNOE000000294718	rPak1 3'UTR	CAATGATGCCCTATTGTCTTGT	TCAGGATCAGCTGCACCAAG	162882023-162882710	688
ENSRNOE000000313488	rPak1 5'UTR	GCCCCCTTCACTGAGCTTCTG	TTCCCTTTCCACAGAAAGGCAG	162817577-162817882	306

Data Handling and Statistical Analysis

Relative quantification, based on a standard curve, was used for QPCR. Mean quantities of triplicate QPCRs for each unknown sample were analyzed using Two-Way ANOVA with $\log_2$ (*Target* quantity/*Rplp2* quantity) as the dependent variable. Independent variables for comparing mammary gland transcript levels were *Mcs3* genotype and DMBA exposure. A two-tailed, unpaired T test was performed *post hoc*. Pearson's correlation coefficients were calculated for *Pak1* and *Ilk* transcript levels from *Mcs3* genotypes. The independent variables were *Mcs3* genotype and DMBA exposure. All statistical analysis was done using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

Results:

Quantitative PCR (QPCR) was used to compare *Ilk* and *Pak1* mammary gland transcript levels between *Mcs3*-resistance associated WF.COP congenic strain D and mammary carcinoma susceptible WF.COP strain E females following DMBA-exposure. A time point of four weeks post-DMBA administration was used. This time represents a stage of early tumorigenesis before frank carcinomas are detectable in a DMBA-induced mammary carcinogenesis model. No effect of *Mcs3* haplotype on expression of *Ilk* or *Pak1* was observed at this time point, which was 12 weeks of age ($p > 0.05$). Expression of *Pak1* was significantly higher in rat mammary glands exposed to DMBA compared to unexposed age matched controls ($p = 0.02$, Figure 6A). There was no effect of either *Mcs3* haplotype or DMBA-exposure on mammary gland *Ilk* transcript levels (Figure 6B). There was a correlation between *Ilk* and *Pak1* mammary gland transcript levels in the mammary cancer

Transcript Levels of *Pak1* and *Ilk* in DMBA-exposed Rat Mammary Glands

Figure 6: Increased Pak1 transcript levels in DMBA-induced rat mammary glands.

RT-qPCR of *Pak1* and *Ilk* levels in rat mammary gland tissue 4 weeks after DMBA-induction of mammary carcinogenesis compared to age-matched uninduced controls. QPCRs, run in triplicate for each sample, were used to measure target gene quantity standardized by *Rplp2* quantity. QPCR Ct values were quantified using linear standard curves derived from known cDNA concentrations. P values are from two-way ANOVA F-tests including exposure and genotype. *Mcs3* genotypes were pooled because genotype was not significant for either *Pak1* or *Ilk*. (**A:** n=21 DMBA rats, 21 control rats; P=0.02. **B:** n=21 DMBA rats, 21 control rats; P=0.47)

Correlation of *Pak1* and *Ilk* Transcript Levels in Rat Mammary Gland Tissue

Figure 7: Correlation of *Pak1* and *Ilk* expression in rat mammary glands is lost with DMBA exposure. Standardized values of *Pak1* and *Ilk* expression from previous RT-qPCR of rat mammary gland tissue (Fig 6) were analyzed by Pearson's correlation. **A:** Rat strain E (susceptible) mammary glands, not exposed to DMBA n=12 control rats, $r=0.1811$, $p=0.5732$. **B:** Strain E age matched rat mammary glands 4 weeks after DMBA-induction of mammary carcinogenesis n=10 DMBA rats, $r=-0.2158$, $p=0.5494$. **C:** Strain D (resistant) rat mammary glands, not exposed to DMBA n=8 control rats, $r=0.9349$, $p=0.0007$. **D:** Strain D age matched rat mammary glands, 4 weeks after DMBA-induction of mammary carcinogenesis n=10 DMBA rats, $r=0.5275$, $p=0.1171$.

resistant WF.COP strain D unexposed control rats ($r=0.9349$, $p=0.0007$, Figure 7C), but this correlation was lost with DMBA exposure ($r=0.5275$, $p=0.1171$, Figure 7D). There was no correlation between *Pak1* and *Ilk* in the mammary cancer susceptible strain E with and without DMBA-exposure.

To determine if genetic variants in *Pak1* protein-coding sequence existed between susceptible and *Mcs3*-resistance associated strains we sequenced WF/NHsd, COP/NHsd, and *Mcs3*- resistance associated WF.COP strain D. This revealed SNVs in exons 1, 2, 11, and 15 that differed between WF and COP or WF.COP strain D, but did not result in amino acid changes (Table 2). One variant was found in the 3' splice site junction of exon 7; however, based on consensus splice sequence, this variant was not predicted to disrupt the splice site. We used the UTRdb database to predict three different upstream open reading frames (uORFs) in the 5' UTR and a polyadenylation signal (PAS) within the 3' UTR of *Pak1*. Variants located in *Pak1* UTRs were not located in these elements. The database miRBase v22 was used to annotate miRNA binding sites within rat *Pak1* UTRs. No sites were identified in the 5'UTR. The 3'UTR contains an *rno-miR-217-5p* site; however, there were no variants between WF and COP or WF.COP strain D at this site.

To determine if the DMBA-induced mammary carcinogenesis model is also a model of PAK1 expression during breast cancer development, IHC was performed on non-cancerous rat mammary tissue exposed to DMBA. Figure 8 contains images of a rat mammary gland that displays positive Pak1 staining compared to an IgG background control. We quantitated relative Pak1 staining in mammary glands again at 4 weeks post-DMBA administration, as this represents an early stage of carcinogenesis. Overall, four out of 17 (24%) DMBA-exposed mammary glands were positive for Pak1 staining; whereas,

Table 2 : Rat Pak1 Sequence Variants between Strains

Marker Type	Type (Structure)	Structure Position (RGSC 6.0/rn6)	Variant Position RNO1	Variant	Reference ¹	WF/Hsd	COP/Hsd	WF.Cop Strain D	Effect on Information Metabolism	Predicted biological impact	SNV ID
SNV	Noncoding (5' UTR)	chr1:162817577-162817795	162817795	G/T	G	T	G	G	None expected	None identified	ss5327331130 ² 14694838 ³
SNV	Coding (Exon 2)	chr1:162830944-162831133	162830964	C/T	C	T	C	C	Synonymous asp>asp	None identified	ss5327331131 ² 14694839 ³
SNV	Splice site	chr1:162860673-162862490	162860683	T/C	T	C	C	C	Synonymous with splice site consensus	None identified	rs106564584 ⁴
SNV	Coding (Exon 11)	chr1:162871744-162871861	162871856	C/A	C	C	A	A	Synonymous arg>arg	None identified	rs106895892 ⁴
SNV	Noncoding (3' UTR)	chr1:162882087-162882685	162882562	C/T	C	C	T	T	None expected	None identified	rs198945644 ⁴

¹Rattus norvegicus strain BN/SsNHsdMCW, ²European Variation Archive (EVA) project accession ID, ³Rat Genome Database ID,

⁴dbSNP ID

Figure 8: Representative Pak1 staining images of morphologically normal DMBA-induced and uninduced rat mammary glands. IHC images of mammary ducts and smaller ductules of a morphologically normal rat mammary gland, 4 weeks after DMBA-induction of mammary carcinogenesis (left two columns) compared to an age-matched rat, not exposed to DMBA (right two columns). Darker staining, relative to the IgG control, is evident in the DMBA-exposed mammary ducts, but not in the uninduced control mammary gland. H&E staining is shown for a histological view of tissue sections. Images were taken at 40x magnification on an Aperio ImageScope CS2.

zero mammary glands out of seven (0%) taken from seven age-matched controls, not exposed to DMBA, stained positive for Pak1 (Table 3). Mammary gland tissue, positive for Pak1 staining at four weeks post-DMBA exposure, appeared histologically normal (Figure 8).

To compare our findings in rat mammary glands with reported results of PAK1 positivity in human breast disease, IHC was used to visualize PAK1 expression in ER+/HER2- ductal carcinoma *in situ* (DCIS) and triple-negative (TN), ER+/HER2-, and HER2+ invasive ductal breast carcinomas (IDC). Images of positive PAK1 staining, relative to IgG control, in TN, ER+/HER2-, HER2+ IDC, and ER+/HER2- DCIS are shown in Figure 9. This was performed as a comparative experiment, with anticipation that our results would recapitulate those reported for PAK1 positivity in human breast disease. One out of six samples stained positive for PAK1 in both TN and HER2+ IDC cases. One out of four ER+/HER2- IDC samples displayed positive PAK1 staining, and one out of two ER+/HER2- DCIS samples were positive for PAK1. Overall, four breast disease tissue samples out of 18 (22%) analyzed were positive for PAK1 staining (Table 4).

Discussion:

We report that a DMBA-induced rat mammary carcinogenesis model of human breast cancer has utility to investigate a potential role for PAK1 at early stages of breast cancer development. Human *PAK1* is located within the breast cancer-associated *11q13/14* amplicon. Human chromosome *11q13/14* amplification is reported to occur in 19.6% of breast tumors and is associated with poor prognosis [244, 277-280, 304]. Functional significance of *PAK1* expression, due to *11q13/14* amplification, has been investigated.

Table 3. Rat mammary glands positive for Pak1 staining

Treatment	Total Samples	Pak1 Positive	Percent Positive
Unexposed controls	7	0	0
DMBA	17	4	24

Figure 9: Images of PAK1 positive staining in human breast tissue. Representative IHC images from (A) ER+/HER2- ductal carcinoma in situ (DCIS) (B) ER+/HER2- invasive ductal carcinoma (IDC) (C) HER2+ IDC and (D) triple negative IDC. Darker PAK1 staining is evident compared to respective IgG controls. H&E staining is shown to provide a detailed view of the tissue. Images were taken at 40x magnification on an Aperio ImageScope CS2.

Table 4. Female breast disease samples positive for PAK1 staining

Breast Disease Subtype	Total Samples	PAK1 Positive	Percent Positive
IDC TN	6	1	17
IDC HER2+	6	1	17
IDC ER+/HER2-	4	1	25
¹ DCIS ER+/HER2-	2	1	50

¹Only a limited number of DCIS samples were available for study

using breast cancer cell lines in tissue culture and xenograft tumors. [288, 289, 305-308]. These *in vitro* studies provide insight into PAK1-driven progression of breast cancer cell lines, but do not indicate if aberrant PAK1 expression possesses a functional role during early disease pathogenesis, as normal tissue transitions toward a carcinoma. We did not uncover evidence supporting a hypothesis that *Pak1* is an *Mcs3* causal gene in this study. There are multiple *Mcs3* nominated genes that will need to be studied in this context [243]. We did discover an independent effect of DMBA exposure on *Pak1* expression in mammary glands. Positive Pak1 expression was detected before pathological signs of disease developed in DMBA-exposed mammary glands. This suggests a potential early functional role of Pak1 in at least a subset of mammary glands suspected to be undergoing carcinogenesis.

We did not measure Pak1 protein and *Pak1* transcript levels in the same samples; therefore, we have neither determined this relationship nor the mechanism for Pak1 positivity in a subset of rat mammary glands exposed to DMBA. We suspect that the increase in *Pak1* transcript levels observed in mammary gland tissue following chemical exposure is due to either repopulation of the mammary gland with a high proportion of Pak1 expressing cells, or increased transcription and/or amplification of the *Pak1* locus in pre-existing cells. Increased mRNA and protein stability are additional mechanisms that will need to be considered to determine the mechanism of Pak1 positivity.

Frequencies of PAK1 positive female breast disease samples found in samples we tested were consistent with a published study of PAK1 staining [282]. We also detected positive PAK1 staining in a human DCIS sample. This matches previous findings of PAK1 positivity in human DCIS lesions using immunohistochemistry [309], and together suggest

PAK1 expression may be involved in the progression of some female invasive breast carcinomas. This is concordant with a transcript profiling study of breast cancer progression that reported higher *PAK1* transcript levels in human DCIS compared to non-diseased breast epithelium [310]. A study of breast cancer cell lines serially passed through xenografts in mice also reported a positive correlation between *PAK1* expression and stage of premalignant progression [311]. Our finding of elevated Pak1 expression in chemical carcinogen-induced non-diseased rat mammary glands further suggests that PAK1 has a role during early tumorigenesis of female breast cancer, potentially prior to the development of premalignant lesions.

Rodent models have long been used to explore gene function during cancer progression [312]. The only published rodent study of PAK1 and breast disease to our knowledge used transgenic mice expressing an activated form of Pak1 [313]. These authors demonstrated a role for Pak1 in mammary lesion and tumor formation; however, the expression of an activated-*Pak1* transgene was dependent upon a pituitary gland isograft. Few mammary tumors developed in this model over a long latency period of 2 years. We propose that laboratory rats, well-known to serve as a female breast cancer relevant *in vivo* system, could be used to study Pak1-mediated mechanisms using a shorter time interval. Palpable mammary tumors typically develop in susceptible rat strains around six-weeks following a single DMBA exposure. A laboratory rat model recapitulates a natural course of breast cancer development, which provides an *in vivo* model to study Pak1-mediated carcinogenesis. The laboratory rat is an excellent model of human breast cancer, as induced rat mammary carcinomas display the same histopathological stages and features of malignant breast carcinomas, including epithelial ductal cell origin, progression, and an

array of hormone responsive and non-responsiveness [76, 101, 160-163, 314]. The mammary carcinoma susceptible rat strains used in this study develop on average 7 mammary tumors per rat upon DMBA induction, which is about 0.58 tumors per mammary gland (MG). Our finding that 24% of DMBA-induced mammary glands were Pak1 positive is extrapolated to estimate Pak1 positivity in 14% of rat mammary tumors (MT) ($0.58 \text{ MT/MG} \times 0.24 \text{ Pak}^+ \text{-MGs}$). This is in line with the 15-25% PAK1 positivity of human breast carcinoma samples in this study, and experimentally demonstrates similar Pak1/PAK1 expression patterns in mammary/breast disease. Therefore, a rat model allows for experimentation and pathway analyses of Pak1 in the context of natural disease initiation, development, and progression that would complement human breast cancer studies.

In this study, we discovered that DMBA-induction of mammary carcinogenesis increased *Pak1* transcript levels in mammary gland tissue appearing morphologically normal. We also reported that Pak1 protein expression in rat mammary glands mirrored what we and others have found in human breast DCIS and IDC samples. These findings of rat/human Pak1/PAK1 staining at early stages of rat mammary carcinogenesis and pre-malignant stages of female breast cancer warrant further functional and diagnostic studies of PAK1 expression during early stages of rat mammary and human breast cancer development.

CHAPTER III

MAMMARY CARCINOMA SUSCEPTIBILITY LOCUS, *MCS3*: FINE MAPPING AND COMPARATIVE GENOMICS

Introduction:

Breast cancer is the most frequently diagnosed form of cancer among women and is the leading cause of premature mortality [315, 316]. It is estimated that 5-10% of familial breast cancer incidences are due to inherited gene mutations such as BRCA1 and BRCA2 [317]. Many of these established breast cancer predisposition, or susceptibility genes, are highly penetrant yet occur at a low population frequency [318]. However, most of the unexplained familial risk and spontaneous disease occurrence is due to a polygenic disease model, where the additive effects of low penetrant variants contribute to increased breast cancer risk [83, 319]. Linkage analysis and candidate gene approaches have historically been utilized to identify high risk breast cancer loci that segregate with breast cancer incidences in families, but they are limited in their ability to identify novel susceptibility genes [65, 320]. Linkage analysis cannot detect the low penetrance alleles that individually confer a small risk on disease development [320] and studies of breast cancer candidate genes are implicitly biased as they evaluate variants in genes with known biological function or involvement in carcinogenic pathways [83, 319]. Their use in identifying genetic factors behind sporadic disease development and the residual heritability of familial breast cancer risk have proved exhaustive.

Genome-wide association studies (GWAS) of breast cancer risk in the general population allows for an objective approach as this method scans the genome for causal variants and does not rely on previous assumptions of biological function or genetic locations [85]. From these studies, a high density of single nucleotide polymorphisms (SNPs) across the genome are identified and mapped as common genetic susceptibility factors, which would have remained unsuspected through linkage analysis and candidate gene approaches [85]. Some of these identified regions contain known genes and indicate new potential pathways with a role in breast carcinogenesis, while others are located in intronic or distal regulatory elements and suggest risk is modulated through regulatory effects [85, 319, 321, 322]. Following identification of variants in risk-associated loci, subsequent fine-mapping and functional studies aim to validate causal SNPs and understand their biological mechanisms [319]. Currently, the causal variants and biological mechanisms underlying these GWAS-identified loci are largely unknown and unannotated [319, 321, 322].

Numerous association studies have been performed but only a small proportion of identified SNPs have true associations with breast cancer, leaving a high false positivity rate. Stringent P values are necessary to lower the false positivity rate, but this also lowers the detection limit and can exclude true risk alleles with very low effect sizes [80]. Furthermore, these studies have only explained a small percentage of breast cancer heritability, indicating there remains considerable breast cancer risk-associated variation to be identified [88]. A comparative genomics strategy employs a model organism in which to genetically identify disease risk loci and evaluate human orthologs of these loci [161]. Integrating combinatorial GWAS and comparative genomics approaches allows for the

discovery of novel risk-associated variants, the identification of true-positive GWAS risk-associations due to overlap in genetic susceptibility, and the ability to mechanistically characterize overlapping breast cancer risk alleles in a model organism that may translate directly to human susceptibility [88, 161, 323].

An extremely useful comparative genomics strategy in studying human breast cancer utilizes a rat model of mammary cancer susceptibility. This consists of mapping mammary cancer risk loci and evaluating human orthologous loci, which may have previously been nominated from independent GWA studies or may have an unidentified role in disease risk [161]. The utility of the rat genome in identifying true positive GWAS-nominated risk associations has been established [88]. One study found that significantly more SNPs from GWA studies mapped to human orthologous regions of rat mammary cancer loci than to human orthologous regions of randomly selected rat genomic regions not known to associate with mammary cancer susceptibility, regardless of the human population used for a GWA study [88]. This study also highlighted that conservative GWAS corrections are too stringent as a considerable number of GWAS-nominated SNPs not meeting genome-wide significance levels had genomic overlap with rat mammary cancer susceptibility loci [88]. This complementary method of risk analysis that integrates rat genomics facilitates validation of variants identified in GWA studies and further studies of these defined orthologous risk loci. Comparative genomics also aids in prioritizing which variants to study amidst the plethora of breast cancer risk associated alleles.

The laboratory rat presents one of the best *in vivo* human breast cancer models as the induced rat carcinoma displays the same histopathological stages and features of malignant breast carcinomas, including epithelial ductal cell origin, progression, and an

array of hormone responsive and non-responsiveness [76, 101, 160-164]. An initial observation found a single oral dose of the chemical carcinogen dimethylbenzanthracene (DMBA) rapidly and reproducibly induces mammary cancer in female Sprague-Dawley rats [212]. Reports followed that outlined differences in carcinogen sensitivity amongst various rat strains. Certain strains displayed a resistance to tumor induction, requiring multiple doses of carcinogen to match the high tumor incidence found in the Sprague-Dawley strain [211]. This finding that inbred rat strains have natural genetic variation in their susceptibility to carcinogen-induced and spontaneous development of mammary cancer led to genetic analysis of mammary cancer susceptibility [232]. Inbred rat strains that differ in their susceptibility to DMBA-, N-methyl-N-nitrosourea (NMU)-, or estrogen-induced mammary carcinogenesis have been used predict locations of quantitative trait loci (QTL) that control susceptibility to mammary carcinogenesis, termed the *Mammary carcinoma susceptibility (Mcs)* and *Estrogen-induced mammary cancer (Emca)* loci [164, 234-239]. In the DMBA mammary cancer model specifically, linkage analysis of segregating populations from crosses between the DMBA-induced mammary carcinoma susceptible Wistar-Furth (WF) and resistant Copenhagen (COP) strains resulted in four predicted *Mcs* QTLs named *Mcs1*, *Mcs2*, *Mcs3*, and *Mcs4* [164, 235].

The existence of *Mcs1*, *Mcs2*, and *Mcs3* QTLs were validated using congenic rat lines carrying the resistant *Mcs* of interest introgressed on a WF susceptible background [159, 243, 324]. These congenic substitutions encompassing the introgressed QTL are tens of megabases in size, so fine mapping of this congenic interval is required to increase the resolution at this locus. Physical mapping can determine if more than one independently acting QTL exists at this locus while also reducing the pool of candidate variants to

functionally characterize [161]. The COP resistant *Mcs3* was physically confirmed and delimited to a 29.4 Mb region on rat chromosome one (RNO1) in 2017 [243]. Here, I report results from fine-physical mapping using WF.COP *Mcs3* congenic strains with differing portions of this COP locus that further delimits *Mcs3* to a 7.2 Mb region on RNO1. I also report findings of breast cancer associated variants using comparative analysis of human orthologous syntenic regions to the narrowed rat *Mcs3*.

Methods:

Congenic breeding:

Congenic rat lines were established and maintained in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility on a 12-hr light/dark cycle and provided LabDiet 5001 Rodent Diet (PMI Nutrition International) and water ad libitum. All animal protocols were approved by the University of Louisville Animal Care and Use Committee. *Mcs3* congenics are defined as genetic lines developed on a WF genome and carrying the selected COP alleles at the *Mcs3* locus. *Mcs3*-associated resistant line D rats have a fixed COP genotype for RN01 *Mcs3* segment from single nucleotide variant (SNV) *rs8149408* to SNV *rs105131702*. Line D males were crossed with inbred Wistar Furth (WF/NHsd) female rats (Envigo). Resulting *Mcs3* heterozygotes were backcrossed to WF females and offspring screened for unique recombination intervals between microsatellite markers near *rs8149408* and *rs105131702*. Novel recombinant rats were backcrossed to WF animals to expand the genotype, and subsequent offspring were inbred by brother-sister mating to establish unique WF.COP-

RNO1 congenic strains for phenotyping. A timeline of this congenic strain development is depicted in Figure 10.

Sequence information and locations of genetic markers defining the ends of COP alleles in the WF.COP *Mcs3* congenics are available at the UCSC Genome Browser (www.genome.ucsc.edu), the Rat Genome Database (<http://rgd.mcw.edu/>), or Table 5 and 6.

Genotyping:

Animals were genotyped as described in Samuelson et al. (2003) [325]. Briefly, a small tail tip section from each rat was removed at 8-11 days of age, lysed, and DNA extracted. Microsatellite marker primers were used to PCR amplify DNA to determine genotypes at the congenic ends of *Mcs3* and at intervals between. Fast-PCR reactions underwent denaturation of 95°C for 10 sec, followed by 40 cycles of 94°C for 0 sec and 63°C for 8 sec, and an extension at 72°C for 30 sec on an Applied Biosystems Veriti Fast Thermal Cycler. PCR products were resolved on 3% high resolution agarose gels, stained with ethidium bromide, scanned using a Typhoon Imager, and visualized with ImageQuant software (Amersham Biosciences, Piscataway, NJ). Visible gel-bands of the appropriate size were analyzed along with DNA from homozygous COP and WF, and (WF x COP)_{F1} (heterozygous) rats. Four *Mcs3* WF/COP informative SNPs were PCR amplified and samples sequenced by the University of Louisville, Center for Genetics and Molecular Medicine, sequencing core using an ABI PRISM 3130XL Sequence Detection System (Life Technologies).

Figure 10: Timeline of Congenic Breeding and Strain Development. The top row indicates rat breedings and generations. The bottom row shows weeks between crosses and new generations. Line D containing the COP *Mcs3* allele is crossed with the WF strain to generate a heterozygous generation. Heterozygotes are bred together to produce a generation of recombinant animals used as founders of new *Mcs3* congenic lines. Founder genotypes are expanded by backcrossing to the WF strain. Brother-sister matings fix these genotypes and establish unique WF.COP *Mcs3* congenic lines. In total it takes about 1 year to develop a new congenic line.

Table 5. Primer Sequences for *Mcs3* Microsatellite Markers in *RN01* used in Screening for Recombinants

Samuelson Lab ID	Forward Sequence	Reverse Sequence	Amplicon Position*	Amplicon size WF/COP (bp)
D1Rat382	GGCCGAATGCTTT CAATAGA	GGCATACATGCTC AAACTGC	137503081- 137503201	123/139
D1Rat173	GATCCCTTGACAA GCATGGT	GATGGAGGCAGTT TTTCCAA	152519382- 152519547	158/174
D1Mit3	ACTTGGTGAAGAA GAGTCAGGG	GATTTACTGTGCC TGTGGTTTT	156446196- 156446783	129/121
D1Rat274	TTGGTACACACAC GCACATG	CTGGACATAAGGA CAACTGGAA	161677683- 161677836	175/157
D1Mgh8	CCTCTGGATTCTG CCAGAAG	TTTCAAATGTACA GGCTGAAACA	163796316- 163796432	143/111
D1Rat277	TCTGGTCTTTACAT GTATGTGCA	TTCACATCAGTTTT GGCCAC	171716519- 171716714	194/210

WF = Wistar Furth, COP = Copenhagen

* *Rattus norvegicus* genome build version RGSC 6.0/rn6

Table 6. Primer Sequences for *Mcs3* SNPs in *RN01*

dbSNP ID	Ensembl ID	Forward Sequence	Reverse Sequence	Amplicon Size (bp)	Amplicon Position*	Variant Position*	Variant	COP	WF
<i>rs8149408</i>	<i>ENSRNOSNP2784088</i>	AGTATCTGC CCGGTGG	GGCAAACCG TCCTGAA	155	143700164- 143700318	143700228	G/A	AA	GG
<i>rs107402736</i>	<i>ENSRNOSNP2784133</i>	TGCTTCGCC TTAACCTG	TGCATGTCA GAAGGGAGA	330	150929449- 150929778	150929594	G/A	GG	AA
<i>rs105307119</i>	<i>ENSRNOSNP2784267</i>	TGACCTCCG TGCTACC	ACCTGGTGT CGCTTCA	253	165593736- 165593988	165593914	T/A	AA	TT
<i>rs105131702</i>	<i>ENSRNOSNP2784324</i>	CTGTCACCC CAGCACT	TGATTTGTCC CGGGGA	281	171517184- 171517464	171517317	T/C	TT	CC

WF = Wistar Furth, COP = Copenhagen

* *Rattus norvegicus* genome build version RGCSC 6.0/rn6

Resequencing:

Intergenic regions at the distal ends of the newly delimited *Mcs3* and approximately the middle of the locus (Chr1:143700228-150929594) were selected to be amplified and sequenced in order to identify additional variants. This locus was searched using rat reference sequence RGSC 6.0/rn6 using UCSC genome browser for regions containing di- and tri-nucleotide repeats. Primers flanking these regions were designed using Primer3 plus oligonucleotide design software and the UCSC genome browser *in silico* PCR tool was used to exclude off target primers. Primers were obtained from Integrated DNA Technologies and used to PCR amplify genomic DNA as described in the previous heading. PCR reactions were run on high-resolution agarose gels to identify potential microsatellite size differences between WF and COP (Table 7).

The genome browser tool JBrowse database v6 as part of the Rat Genome Database was used to search for informative variants. The WF/NHsd genome is not sequenced, so the COP/CrCrl genome was compared to the Lewis (Lew/Crl), Milan Hypertensive Strain (MHS/Gib), and inbred Wistar (WN/N) genomes. Lewis and MHS are derived from Wistar stock, and together with the Wistar strain have similar genetic backgrounds as the WF/NHsd strain. Primers were designed to amplify a region roughly 150-300 bp in size that encompass the SNP. These regions were PCR amplified at cycling conditions of 94°C for 2 minutes, 18 cycles of 94°C for 15 seconds and 68°C for 2 minutes, 22 cycles of 94°C for 15 seconds and 59°C for 2 minutes, and 59°C for 10 minutes. PCR reactions were cleaned up using ExoSAP-IT (Thermofisher) at cycling conditions of 37°C for 15 minutes and 80°C for 15 minutes. Samples were then Sanger sequenced using an ABI PRISM 3130xl Genetic Analyzer (Table 9).

Phenotyping:

Female WF.COP-RNO1 congenic rats, along with female WF/NHsd rats (Envigo), as positive (susceptible) controls, were administered a single oral gavage of DMBA (65 mg DMBA/kg body mass)(ACROS Organics; Fisher Scientific, Pittsburgh, PA) in sesame oil at 50–55 days of age. Mammary carcinomas $\geq 3 \times 3 \text{ mm}^2$ were counted at 15 weeks post-treatment. N=28 WF, 20 line H, 26 line I, 30 line J, and 25 line K.

WF.COP Line J congenic rats and WF/NHsd rats (Envigo) were maintained in facilities described in congenic breeding. Rats were weighed at 4, 8, and 12 weeks of age (N=31 WF males, N=34 WF females, N=30 line J males, and N=29 line J females). Separate female WF.COP Line J congenic rats and WF/NHsd rats (Envigo) were weighed at 4 weeks of age (weaning), then placed into a room of only female housing. Rats were again weighed at 8 and 12 weeks of age (N=15 WF females, N=24 line J females).

Tumor Collection and Hematoxylin and Eosin Staining:

Representative mammary tumors (invasive ductal carcinomas) were resected from females from each WF.COP-RNO1 congenic line and WF/NHsd rats (Envigo) at 15 weeks post-DMBA administration. Tumors were formalin fixed and paraffin embedded (FFPE). FFPE blocks were cut into 4 μm sections and placed onto Superfrost Plus microscope slides using a rotary microtome (Thermo HM 355S). Slides were deparaffinized and rehydrated using 3 separate xylene washes and 4 washes in 100% ethanol, 90% ethanol, 80% ethanol, and DI water. Tissue was stained with hematoxylin (Fisher Scientific 469803) for 3 minutes, rinsed in running DI water, dipped in bluing reagent (Fisher Scientific 22-220-106), and rinsed in running DI water. Tissue was stained with Eosin-Y (Fisher Scientific 22-220-104) for 30-45 seconds. Tissue was dehydrated in washes of 90% ethanol, 100%

ethanol, and 3 separate xylene washes. Tissue was coverslipped with Permount Mounting Medium (Electron Microscopy Sciences 17986-05)

Statistical analysis:

Mammary carcinoma multiplicity data were analyzed by comparing congenic strain phenotypes to a susceptible WF/NHsd phenotype. Mammary carcinoma multiplicity phenotypes for all lines were compared using a one-way ANOVA. Following a significant one-way ANOVA (p value < 0.05), each congenic strain was compared to the WF/NHsd susceptible control group using Dunnett's multiple comparison test. P values ≤ 0.05 were considered statistically significant. Body mass data were analyzed by a 2-way ANOVA using strain and age as the independent variables. Tukey's test for multiple comparisons was used *post hoc*.

Comparative genomics:

Human and mouse orthologous syntenic regions that mapped to the delimited rat *Mcs3* were identified using the convert function of the UCSC Genome Browser. The *Rattus norvegicus* reference genome build version RGSC 6.0/rn6, *Homo sapiens* version GRCh38/hg38, and *Mus musculus* version GRCm38/mm10 were used. Rat *Mcs3* and human and mouse orthologous syntenic regions were annotated for genes and non-coding DNA using the table browser function of the UCSC Genome Browser. The NHGRI-EBI Catalog of human genome-wide association studies was used to identify breast cancer disease and associated traits that had p values of 1×10^{-3} or less in the human syntenic regions. PubMed was used to data mine *Mcs3* and human orthologous genes associated with cancer.

Results:

I. Fine Mapping *Mcs3* Using WF.COP Congenic Rat Lines

Backcrosses of WF.COP *Mcs3* line D result in novel WF.COP *Mcs3* congenic lines

Mcs3 was previously predicted as a DMBA-induced mammary cancer susceptibility QTL by Michael Gould's group, and was physically confirmed and delimited to a 29.4 Mb region on rat chromosome 1 by *Le et al* in 2017 [164, 243]. I sought to further map the *Mcs3* QTL to a shorter interval to reduce the pool of candidate genes and variants that could be modulating mammary cancer susceptibility. To accomplish this fine mapping, an additional four congenic lines were derived from the original Copenhagen (COP) locus in WF.COP *Mcs3* Line D. These were termed lines H, I, J, and K and possessed a unique portion of the COP introgression on chromosome 1 from the previously defined *Mcs3* on a susceptible WF genetic background (Fig 11). The genomic location and positional markers defining the COP region of these lines are given in Table 7.

Mammary carcinoma susceptibility phenotypes of WF.COP congenic lines delimit *Mcs3* to a 7.2 Mb region on rat chromosome 1 (Chr1:143700228-150929594)

The new WF.COP *Mcs3* congenic lines were tested for DMBA-induced mammary carcinoma susceptibility phenotypes in order to further delimit the *Mcs3* QTL. Tumor multiplicity data for each WF.COP *Mcs3* congenic lines depicted in Figure 11 is shown in Figure 12 and quantified in Table 9. Tumor multiplicity is counted 15 weeks following administration of DMBA and represents the DMBA-induced mammary carcinoma susceptibility phenotypes. WF females were administered DMBA alongside WF.COP

Figure 11. WF.COP congenic map of *Mcs3* region recombinants used as congenic line founders. The leftward black bar represents the previously delimited *Mcs3*, defined by a COP segment (black) of *RNO1* introgressed into a WF/NHsd strain genetic background. Horizontal lines along the axis represent informative markers used to distinguish WF and COP alleles along the *Mcs3* locus. The remaining black bars represent the retained COP segments in the congenic lines used to narrow *Mcs3*. Gray bars at the ends of congenic segments mark regions of unknown genotype.

Table 7. Positional markers and genomic locations defining WF.COP *Mcs3* congenic lines

WF.COP <i>Mcs3</i> line	Congenic region marker/marker	Genomic position*	Size (Mb)	Percent of <i>Mcs3</i> span
H	D1Rat274 to D1Rat277	Chr1:161677683-171716714	10	34
I	rs107402736 to D1Rat277	Chr1:150929594-171716714	20.8	71
J	D1Rat382 to D1Rat274	Chr1:137503201-161677683	24.2	61
K	D1Mit3 to D1Rat277	Chr1:156446196-171716714	15.3	52

* *Rattus norvegicus* genome build version RGSC 6.0/rn6

Mcs3 congenic lines to serve as controls for a susceptible phenotype and developed an average of 7.9 ± 2.9 (mean $\pm$ SD) tumors per rat. The susceptible WF phenotype in this study was similar to previously published DMBA-induced susceptibility phenotypes in WF females. *Veillet et al.* reported an average of 7.9 ± 0.5 tumors per WF rat, and *denDekker et al.* reported WF rats developed 6.3 ± 3.3 tumors per rat upon DMBA induction [243, 248, 326]. Line H, I, and K displayed average high mammary tumor numbers per rat similar to the WF strain of 6.7 ± 3.4 , 5.9 ± 3.8 , and 7.0 ± 3.5 respectively. Line J showed a lower average of 3.4 ± 2.7 tumors per rat. Tumor multiplicity data was analyzed by a statistically significant One-way ANOVA ($p < 0.0001$), followed by Dunnett's nonparametric *post hoc* tests to compare each congenic line to the WF susceptible control. Line J was the only line to display a DMBA-resistant phenotype as it was significantly different from the WF strain. The resulting mammary carcinoma phenotypes and positional maps of the original founder lines and congenic lines in this study are depicted in Figure 13. The overlap of the susceptible lines do not indicate that there are independently acting *Mcs* sub-QTLs. Taken all together, the congenic lines delimit rat *Mcs3* to a 7.2 Mb region on rat chromosome 1 that spans from *rs8149408* to *rs107402736* (Chr1:143700228-150929594).

To further fine map the newly delimited *Mcs3*, the rat genome database (RGD) was queried for microsatellite markers in this region but reported markers did not differ in size between WF and COP alleles and were thus not informative. To uncover additional microsatellite markers within *Mcs3*, intergenic regions of the middle segment and distal ends of the newly delimited *Mcs3* spanning from markers *rs8149408* to *rs107402736* were scanned using the UCSC genome browser to find short genomic regions rich in di- and tri-nucleotide repeats within this interval. A total of 33 of these potentially

Figure 12: Tumor Multiplicity of WF.COP *Mcs3* Congenic Lines. Shown are the distribution of tumor count data of congenic line H, I, J, and K alongside the WF strain as a susceptible phenotype control. Tumors were counted at 15 weeks post-DMBA administration when rats were 23 weeks of age. A Kruskal-Wallis test was performed ($p < 0.0001$), followed by Dunn's nonparametric post hoc tests comparing each line to the control WF strain. Line J was the only line that significantly differed from the WF strain in tumor multiplicity ($p < 0.0001$).

Table 8. Tumor Multiplicity Summary Data of *Mcs3* WF.COP Congenic Lines

Line	Congenic region marker/marker	Mean (SD) Mammary Carcinomas <i>per</i> Rat	N	p-value against WF/NHsd
D	D1Rat320/D1Rat65	2.8 (2.3)	19	-
H	D1Rat274/D1Rat277	6.7 (3.4)	20	0.7666
I	rs107402736/D1Rat277	5.9 (3.8)	26	0.0932
J	D1Rat382/D1Rat274	3.4 (2.7)	30	<0.0001
K	D1Mit3/D1Rat277	7.0 (3.5)	25	>0.9999
WF/NHsd	-	7.9 (2.9)	28	-

WF.COP *Mcs3* Congenic Map

Figure 13. WF.COP Congenic Map Defining the Genomic Position of *Mcs3*. Vertical bars define the COP segment of RNO1 introgressed into a WF/NHsd inbred strain genetic background. Red colored bars represent congenic lines that display a resistant phenotype, yellow bars represent a susceptible phenotype, orange bars represent a mixed phenotype, and grey bars represent segments of unknown recombination where the genotype wasn't determined. The leftward lines D and E are from the previous physical mapping of *Mcs3* and represent how this locus was delimited. Congenic lines on the right are derived from line D and further narrow this locus (rightward black bar).

polymorphic microsatellite markers were tested by designing flanking primers, amplifying the region, and running PCR products on high-resolution agarose gels (Table 9). However, none of these were informative between WF and COP genotypes. After failing to discover new *Mcs3* microsatellite markers, the RGD genome browser tool JBrowse database v6 was searched for differing variants between genotypes. Since the WF/NHsd genome is not available, the COP/CrCrI genome was compared to the Lewis (Lew/CrI), Milan Hypertensive Strain (MHS/Gib), and inbred Wistar (WN/N) genomes. Lewis and MHS are derived from Wistar stock, and together with the Wistar strain have similar genetic backgrounds as the WF strain. Within *Mcs3*, 20 SNPs were identified that reportedly differed in the COP strain. After designing primers and sequencing across these variants, it was found that none of these SNPs differed between COP and WF (Table 10). This inability to find informative markers between WF and COP suggest that they share similar haplotypes at *Mcs3*. As a result, I stopped searching for markers in this tedious manner, so *Mcs3* was unable to be mapped to a shorter genomic interval than what was found from the development and phenotyping of our congenic lines.

WF.COP *Mcs3* congenic lines have differing mammary tumor multiplicities but share similar tumor histopathology

To determine if a difference in tumor histopathology was associated with the observed differences in tumor multiplicity, mammary tumor sections from WF and each WF.COP *Mcs3* congenic line were stained by hematoxylin and eosin (H&E). The DMBA-induced mammary tumors from each line were all of ductal origin, with the majority tumor histological subtypes being invasive papillary carcinoma (Fig 14) and invasive cribriform

Table 9. Primer Sequences Tested for New *Mcs3* Informative Microsatellites

Samuelson Lab ID	Forward Sequence	Reverse Sequence	Chr 1 Position*
A186_gULRN01_1	AGGCTCTCCTTTC TACCTCTGT	GGTGGGCGTACACT TTAGGT	145501208-145501435
A186_gULRN01_2	TGAGCTAAGGAT GAGGGGCT	TCCTGTCCTCAAAT GCCAC	145506302-145506564
A186_gULRN01_3	ATACAGGCACAT GCTTCGTG	ACATCAAATCAAA GCAAAATCGATCA	145509109-145509341
A186_gULRN01_4	CCAGATCAGTGA GAGGCTCC	GAACATCCCACCAC TGCCAG	145510601-145510801
A186_gULRN01_5	TGTGATACCCTCT TCTGGTGTG	CACCTAAGGTAGCG AAAGAGCA	145516753-145516980
A186_gULRN01_6	AGACAGACAAAT CTTAAGGGACCA	TGCTTTAGTACCAA GGATCTGAGT	145532420-145532725
A186_gULRN01_7	CGCATGTGACTG GAGGAGAA	GAGAGCATATAGG TTCCACCCA	145534867-145535141
A186_gULRN01_8	CCTCTGATGCCCA CATGTGT	TGTGTGTATGTGCA TGTAGGT	145538047-145538198
A186_gULRN01_9	TCAAGGAGGTTT TCAGTTGTGA	CTTGCAGCTCTGGG GATCAA	145545762-145545974
A186_gULRN01_10	GCATGAGCTTTCT AATTCACACTGA	AGAGAAGATCCCG CTAGGTCT	145549788-145549991
A186_gULRN01_11	TCTCCCCCATCCA AATCACA	GGGAGGAAGGGAG GACAGAA	145559506-145559729
A186_gULRN01_12	ACCTTGACTCCAA AGCCAAT	GGCTCAAGCTTGCA ACAGTT	145575831-145575998
A186_gULRN01_13	AAGTCCAGGCAG AGTGAGGA	AGTCACAGAAAGG AGCATGGA	145576351-145576497
A186_gULRN01_14	CCTCCCTGGCCCA GTAATTC	GCAGGAGGGAGAA GTGTTCC	145581088-145581249
A186_gULRN01_15	CTCACAGAGCTTC CATCCCC	GAGGGAAAAGGGA CAGTGGG	145588803-145588952
A186_gULRN01_16	TGAGTCTGCATTG TGGGTGC	AGTTTCAGTGAGAG ACTCCGTATT	145591580-145591793
A186_gULRN01_17	AAGCCAAGCAAA TAAATTTTCATTCA	GGCAGTAGCTACAC CATGGA	145595659-145595873
A186_gULRN01_18	AGTCTGTGAGGA TGGTCCCT	TGCAGAGAACAAT GAGATGGCT	145605424-145605629
A186_gULRN01_19	ATATTCCAGACTT GCCTACAGG	CTCCCCTCAGTCCC TGTGTT	145626825-145626998
A186_gULRN01_20	CCTTCTGGCAACT AAGGGCA	TGGAATTCTTAAC ATGAGATGAGATG	145638005-145638182
A186_gULRN01_21	GGGTCGTTACAG AGCGGTG	CCAGTGGGCAAGG GAGGC	147112809-147113012
A186_gULRN01_22	CCTTTCTTGTGGC TTTTGGGT	TCCAAATGGGCAA ATGGTTTGA	146907551-146907671
A186_gULRN01_23	TGGTACACATAT ATGCCACCCA	TCTGGATAACCAAC TTAATGGCA	146908208-146908398
A186_gULRN01_24	TGTTTAGTATGCA TGGAGCCCT	TCATGTTCTTTATTT ACAAGCCTAACA	146854985-146855162
A186_gULRN01_25	GGTATGGGCTTGT TGGAGGT	CTGGAGGCATAGCT GGGAG	146856680-146856848
A186_gULRN01_26	GACCCAATGGCC ATTTTCTCTG	GGGAGGCTCTGCTC AATGTT	146867455-146867635

A186_gULRN01_27	GCTGGACGGTTG TGTTAGCT	GTTGTACACTGGCC TCCACA	146872229-146872360
A186_gULRN01_28	TGGGGCAGAGTT AACACAGAC	TCCTTCAAGACTGT ACCCTGT	148319017-148319217
A186_gULRN01_29	AGCCATAGAGGT CAGGAAGA	CCTGAGATTTACAC TCACAGCA	148330186-148330327
A186_gULRN01_30	ACAAGCTTGTTTT CAGAATTTCTGT	TGCATTCCCAAGAT TACATGCT	148363769-148363904
A186_gULRN01_31	CTCGCAAGCACA CCAAAATCT	TCAGGTCCAGTAGT TTTTCAGGT	148387678-148387840
A186_gULRN01_32	TCCCCTCAGAAG AAACCCCT	ACCCTAGAGTATAG AGTAGAGGAGA	148267287-148267422
A186_gULRN01_33	TGTTTTCTAAACT GCTTTCACCTGT	TGTGACCATAGGTA TGTGGGT	148287640-148287840

* *Rattus norvegicus* genome build version RGSC 6.0/rn6

Table 10. SNP Markers Tested in WF and COP Rats Based on Published Data in the *Mcs3* Interval

Samuelson Lab ID	Public ID	Forward Primer Sequence	Reverse Primer Sequence	Chr 1 Position*	BN (reference)	WF	COP
A176_g01UL1	-	ACTGTGAAGGGAATA CACCAGG	TTTTCCTGTGCTGA GCTGGC	147,298,146/147,298,156	C/C	-	-
A176_g01UL2	-	ACACAGGTAAACATGT AGAAGCCA	TGAATGTTTGGAT TAGGACGTTT	147,286,475/147,286,604	G/A	A/T	A/T
A176_g01UL3	-	ACCTAATGATCCAGAA ATACCACTCT	GCAAAACTCAGGA TGTGCTCA	147,294,091/147,294,151	C/C	A/T	A/T
A176_g01UL4	-	AAGGTTCTGAGCCGAT GCAA	AATTGTGGCAGGTC TTGGGG	147,294,864/147,294,865	G/G	-	-
A176_g01UL5	rs105823832	TCTACCCACACACACC CAGA	TGGAGTACAGGAT ATGCTTCCC	147,267,291	C	-	-
A176_g01UL6	rs105018076	CTCCACTTGATCCCCA CCCT	TGCAGAAAGGGGAT TGTAGCA	147,267,515	T	C	C
A176_g01UL7	rs65980570	CCTCTCCAAGAGTGTT GCAC	AAAGGTCACTTGG GAAGCC	146,437,110	T	C	C
A176_g01UL8	rs106392170	ACCTCACTTCTGTTC ACCCA	CTGTCACTACCCCC TCCTCT	146,458,204	G	A	A
A176_g01UL9	rs105240078	ATAACAGTAACAGCCC TGAATATGC	AATTGTTTCAACTG GAAATGAGTTGG	146,482,838	T	C	C
A176_g01UL10	rs198960055	CTGCGGGCATAACGTAT CTGT	GCTCACAGAAAGGC CACAGAT	146,551,551	C	T	T
A176_g01UL11	rs198339413	AGCCTAGGGTAGTGGT GGTC	TGGTGAAGCAAGT GATGGCA	146,577,774	T	G	G
A176_g01UL12	rs104962053	TCACTAGGGATCTGAA CTCAGGA	TGAAGACACACAC ACACCCA	146,655,635	A	G	G
A176_g01UL13	rs105789263	AAGCCTTCCTTGGTCC CATG	CATGGAGGTAGGG TTTGTGGT	146,663,903	A	G	G
A176_g01UL14	rs105408745	ACGGACTAGTATAATA CTAAGTGGGG	GAATCACAGAATG AGCAAGCAGG	146,693,396	T	C	C
A176_g01UL15	rs107436795	ATTCCATGAGCTTGGT CACA	CATTCACTCCCCAG GCTTCT	146,731,392	T	C	C
A176_g01UL16	rs197249232	CCATCAGATAGGGCTG TGGC	ACATCCCCTCCAAG GATCAGA	146,842,521	T	G	G

BN = Brown Norway, WF = Wistar Furth, COP = Copenhagen **Rattus norvegicus* genome build version RGSC 6.0/rn6

carcinoma (Fig 15). Thus, each line appeared to develop invasive ductal carcinomas with no discernable differences in tumor histopathologies between lines.

***Mcs3* has a pleiotropic effect on mammary tumor susceptibility and body mass which is not due to a delay in mammary gland development**

In addition to mammary tumor susceptibility, there was a pleiotropic effect of *Mcs3* on body mass. At 15 weeks post-DMBA administration there was a significant difference in body mass in line J females as compared to the other *Mcs3* congenic lines and WF strain (Fig 16). Lines K and H displayed an average body mass of 210 g, WF showed an average of 201 g, and line I showed an average of 202 g. Line J was significantly lower with an average body mass of 179 g (one-way ANOVA followed by Dunnett's *post hoc* test $p < 0.0001$) (Table 11). Body mass data collection was extended to the regular breeding colonies of WF and line J to determine if this lower body mass phenotype was solely attributable to the effects of DMBA. Weights were recorded at timepoints of 4, 8, and 12 weeks of age in order to capture stages of development (weaning age, puberty, and breeding age) (Fig 17) (Table 12). There was no significant difference between lines or sex at 4 weeks of age. There was a significant difference between WF males and line J males at 8 weeks of age, with WF weighing an average of 186g and line J weighing 150g (2-way ANOVA followed by Tukeys *post hoc* test $p < 0.0001$). There was also a significant difference between WF and line J males at 12 weeks of age, with WF displaying an average body mass of 274g and line J displaying 244g (2-way ANOVA followed by Tukeys *post hoc* test $p < 0.0001$). Contrasting with the body mass data from DMBA females, line J

Figure 14: WF.COP *Mcs3* congenic lines develop invasive papillary carcinomas. H&E staining of DMBA-induced mammary tumors from WF strain and derived congenic lines (columns). The top and bottom rows show differing magnifications. The papillary pattern is defined by fibrovascular cores that form a network throughout the tumor (arrow A), with neoplastic epithelial cells that grow and expand the lesion via papillary projections (arrow B). Images were taken at 40x magnification on an Aperio ImageScope CS2.

Figure 15: WF.COP *Mcs3* congenic lines develop invasive cribriform carcinomas.

H&E staining of DMBA-induced mammary tumors from WF strain and derived congenic lines (columns). The top and bottom rows show differing magnifications. The cribriform pattern is defined by neoplastic epithelial cell tumor nests (arrows A) within a surrounding desmoplastic stroma (arrow B). Images were taken at 40x magnification on an Aperio ImageScope CS2.

Figure 16: Body mass of *Mcs3* experimental females at 15 weeks post-DMBA administration. The WF strain and *Mcs3* congenic lines were weighed at 23 weeks of age at the time of tumor multiplicity counts. A one-way ANOVA was performed ($p < 0.0001$), followed by Dunnett's *post hoc* tests comparing each line to the control WF strain. Line J was significantly different from WF ($p < 0.0001$).

Table 11. Body Mass of *Mcs3* WF.COP Congenic Females 15 weeks Post-DMBA Administration

<i>Mcs3</i> WF.COP Line	Mean (SD) weight (g)	N	p-value against WF/NHsd
H	210 (11)	18	0.9560
I	202 (13)	26	>0.9999
J	179 (17)	28	<0.0001
K	210 (13)	25	0.4788
WF/NHsd	201 (21)	26	-

Figure 17: Body mass of WF and Line J rats over development. Male and female rats of each strain were weighed at weaning age (4 weeks), puberty (8 weeks), and breeding age (12 weeks). There was no significant difference in body mass at 4 weeks of age or in any age comparing WF or line J females. WF and line J males had significantly different weights at 8 and 12 weeks of age (2-way ANOVA followed by Tukeys multiple comparisons post hoc test $p < 0.0001$).

Table 12. Body Mass Summary Data of WF/NHsd and WF.COP Line J Breeders

Strain	Sex	Housing	Age (weeks)	Mean (SD) weight (g)	N
WF/NHsd	Male	Regular	4	67 (11)	31
WF/NHsd	Male	Regular	8	186 (15)	27
WF/NHsd	Male	Regular	12	267 (29)	37
WF.COP Line J	Male	Regular	4	52 (6)	17
WF.COP Line J	Male	Regular	8	149 (21)	28
WF.COP Line J	Male	Regular	12	244 (37)	29
WF/NHsd	Female	Regular	4	65 (11)	22
WF/NHsd	Female	Regular	8	130 (8)	31
WF/NHsd	Female	Regular	12	175 (14)	38
WF.COP Line J	Female	Regular	4	51 (8)	20
WF.COP Line J	Female	Regular	8	122 (13)	29
WF.COP Line J	Female	Regular	12	168 (18)	23
WF/NHsd	Female	Female Only	8	134 (5)	15
WF/NHsd	Female	Female Only	12	178 (7)	15
WF.COP Line J	Female	Female Only	8	114 (9)	24
WF.COP Line J	Female	Female Only	12	152 (13)	24

females in general housing displayed no significant difference in weight from the WF strain. Since DMBA females are kept in a separate room that only houses female rats, WF and line J females (not exposed to DMBA) were moved to this room and body mass data re-collected (Fig 18). This time there was a significant difference between WF and line J females at 4 weeks of age (65g vs 50g), 8 weeks of age (134g vs 113g) and 12 weeks of age (178g and 152g) (2-way ANOVA followed by Tukeys *post hoc* test $p < 0.0001$).

To explore if reduced body mass was reflected as a delay in mammary gland growth and development, mammary glands were collected and H&E stained from WF and line J females at 4, 8 and 12 weeks of age (Fig 19). Line J and WF females displayed similar morphologies of mixed terminal end buds (TEBs) and ductal structures at 4 weeks of age. At 8 weeks of age, both rat lines displayed a mammary gland histology of mostly TEB structures. Adult line J and WF rats at 12 weeks of age exhibited a largely ductal histology. There were no discernable differences in gross mammary gland histology or morphological structure between rat lines at these developmental timepoints.

II. Human Orthologs to the Newly Delimited Rat *Mcs3*

Comparative genomics identifies human syntenic regions to rat *Mcs3* with orthologous genes

Using the convert genomes function in the UCSC Genome Browser, the newly delimited rat *Mcs3* (7.2Mb) was found to align to three orthologous human syntenic regions on chromosomes 11 and 15 (Table 13). The July 2014 rat assembly (build 6.0/rn6) and December 2013 human assembly (build GRCh38/hg38) at the UCSC Genome Browser

Body Mass by Age of WF and WF.COP Line J Females Raised in all Female Environment

Figure 18: Body mass of WF and Line J females raised in an all-female environment.

Female rats of each strain were kept in all female housing and weighed at 4, 8, and 12 weeks of age. There was a significant effect of strain on body mass at all stages of development (2-way ANOVA $p < 0.0001$).

Figure 19: Rat mammary gland histology in WF and line J females over development.

H&E staining of mammary glands, top and bottom rows display different magnification. Images were taken at 40x magnification on an Aperio ImageScope CS2. **A:** mammary gland morphology is a mix of ductal and terminal end bud (TEB) epithelial structures embedded throughout the adipocyte matrix at the start of puberty in both WF and line J. **B:** TEB structures have reached maximal numbers during puberty and appear as the predominant morphology in WF and line J females. **C:** TEB structures are no longer readily apparent in the adult mammary gland. Greater developed ductal structures within a more pronounced fibrous stroma appear as the major epithelial morphology in WF and line J females.

table browser function were used to annotate rat *Mcs3* and human syntenic regions. A list of genes encompassed in these regions are given in table 13, and non-coding DNA given in Table 15. Orthologous syntenic regions to rat *Mcs3* were also annotated in the mouse genome (December 2011 assembly, build GRCm38/mm10). This was done to potentially place human syntenic genes that were not annotated in rat *Mcs3* since the human and rat genomes are more divergent than human and mouse. However, no genes or non-coding DNA was shared exclusively between the mouse and human syntenic regions. Overall, 23 orthologous genes were found in common between rat *Mcs3* and human syntenic regions, with 12 genes annotated in the human only and 20 in the rat only. A majority of these rat-only genes encode olfactory receptors not found in the human. There are also 30 lncRNAs encoded in rat *Mcs3* and 16 lncRNAs and 4 miRNAs in the human syntenic regions.

Human orthologous genes and variants have associations with cancer and body mass traits

PubMed was used to data mine *Mcs3* and human orthologous genes associated with cancer, which are listed in Table 16. The human orthologous syntenic regions were also analyzed for breast cancer disease and associated traits using the NHGRI-EBI Catalog of human genome-wide association studies. Two of the human syntenic regions at chr15:80005820-82285404 and chr15:83134545-84130720 possessed 72 relevant variants. Table 16 lists overlapping GWAS SNPs and associated traits within these regions, which include breast cancer specific mortality in ER+ breast cancer, age to menarche, body mass index, waist-hip ratio, fat body mass, body height, lean body mass, BMI-adjusted hip circumference, BMI-adjusted waist circumference, body fat distribution, body fat

Table 13. Orthologous Human Loci Syntenic to Rat *Mcs3**

Human chromosome position (GRCh38/hg38)	Percent of bases	Percent of span
chr15:80005820-82285404	12	31.6
chr15:83134545-84130720	4.4	13.2
chr11:89365341-89617253	1.1	4.2

**Mcs3* position Chr1:143700228–150929594 (assembly RGSC 6.0/Rn6)

Table 14. List of Genes and Genomic Position in Rat *Mcs3* and the Orthologous Human Regions

Rattus norvegicus (RGSC 6.0/Rn6)			Homo sapiens (GRCh38/hg38)		
Gene	Accession number	Genomic Position	Gene	Accession number	Genomic Position
Tm6sf1	NM_001108490.1	Chr1: 143657606-143703759	TM6SF1	NM_023003.5	Chr15: 83107644-83137408
Hdgfrp3	NM_145785.1	Chr1: 143702863-143751789	HDGFL3	NM_016073.4	Chr15: 83127752-83207823
Bnc1	NM_001108916.1	Chr1:143869439-143878077	BNC1	NM_001717.4	Chr15:83255883-83284664
Sh3gl3	NM_031238.2	Chr1:144069640-144200030	SH3GL3	NM_003027.5	Chr15:83447340-83618743
Adamtsl3	NM_001107533.2	Chr1:144238957-144561065	ADAMTSL3	NM_207517.3	Chr15:83654122-84039842
Saxo2	NM_001106277.1	Chr1:144586259-144601327	SAXO2	NM_001348699.2	Chr15:82262817-82284927
Efl1	NM_001107534.1	Chr1:144601424-144740634	EFL1	NM_024580.6	Chr15:82130232-82262734
Mex3b	NM_001191626.1	Chr1:144831522-144835758	MEX3B	NM_032246.6	Chr15:82041777-82046018
Tmc3	NM_001170432.1	Chr1:145715968-145760623	TMC3	NM_001080532.3	Chr15:81331087-81374213
Stard5	NM_001192011.1	Chr1:145770134-145780001	STARD5	NM_181900.3	Chr15: 81309052-81324141
Il16	NM_001105749.1	Chr1:145781927-145870912	IL16	NM_004513.6	Chr15:81296969-81314058
Cfap161	NM_001100581.1	Chr1:145907092-145931583	CFAP161	NM_173528.4	Chr15:81134269-81149179
Mesdc1	NM_001013149.1	Chr1:146027820-146029840	TLNRD1	NM_022566.3	Chr15:81000922-81005788
Mesdc2	NM_001008345.1	Chr1:146037425-146043097	MESD	NM_015154.3	Chr15:80975751-80989819
Abhd17c	NM_001100736.1	Chr1:146248504-146289465	ABHD17C	NM_021214.2	Chr15:80695309-80755621
Arnt2	NM_012781.3	Chr1:146399216-146556437	ARNT2	NM_014862.4	Chr15:80404381-80597933
Fah	NM_017181.2	Chr1:146713662-146736339	FAH	NM_000137.3	Chr15:80152998-80186949
Zfand6	NM_001007630.1	Chr1:146745849-146823762	ZFAND6	NM_019006.4	Chr15:80059675-80138393
F8a1	NM_001109323.1	Chr1:147021435-147022950	F8A3	NM_001007524.1	ChrX:155457484155458600
Cyp2c6v1	NM_001013904.1	Chr1:147713900-147814395	CYP2C19	NM_000769.4	Chr10:94762680-94855547
Cyp2c7	NM_017158.2	Chr1:147422998-148119857	No human ortholog		
Mpp1	NM_001037659.1	Chr1:148450212-148458945	No human ortholog		
Folh1	NM_057185.2	Chr1:150323767-150395415	FOLH1	NM_004476.3	Chr11:49145091-49208602
Nox4	NM_053524.1	Chr1:150797201-150974737	NOX4	NM_016931.5	Chr11:89324352-89491375
Cemip	XM_218855.9	Chr1:146069773-146226320	CEMIP	NM_018689.3	Chr15:80779369-80951771
Vbp1	XM_006229645.3	Chr1:148425045-148443505	No human ortholog		

Vom2r40	NM_173317.1	Chr1:148927531-148974509	No human ortholog
Vom2r42	NM_001099511.1	Chr1:149132319-149175214	No human ortholog
Vom2r43	NM_001099512.1	Chr1:149506821-149529350	No human ortholog
Olr12	NM_001000540.1	Chr1:149578356-149579280	OR13G1 NM_001005487.1 Chr1:247672117-247673041
Olr16	NM_001000694.1	Chr1:149674492-149675443	No human ortholog
Olr17	NM_001000937.1	Chr1:149682400-149683357	No human ortholog
Olr13	NM_001000116.1	Chr1:149786381-149787308	No human ortholog
Olr14	NM_001000695.1	Chr1:149816123-149817065	No human ortholog
Olr19	NM_001000117.1	Chr1:149913646-149914606	No human ortholog
Olr20	NM_001000118.1	Chr1:149963546-149964539	No human ortholog
Olr23	NM_001000119.1	Chr1:150067241-150068237	No human ortholog
Olr24	NM_001000693.1	Chr1:150100235-150101177	No human ortholog
Olr25	NM_001000692.1	Chr1:150130083-150131076	No human ortholog
Olr27	NM_001000936.1	Chr1:150172374-150173316	No human ortholog
Olr29	NM_001000691.1	Chr1:150209395-150210403	No human ortholog
Olr30	NM_001000120.1	Chr1:150225372-150226302	No human ortholog
Olr32	NM_001000690.1	Chr1:150261205-150262213	No human ortholog
Olr34	NM_001000689.1	Chr1:150310318-150311257	No human ortholog
No rat ortholog			CTXND1 NM_001352888.2 Chr15:80195480-80252213
No rat ortholog			OR6A2 NM_003696.2 Chr11:6794524-6795908
No rat ortholog			TWNK NM_021830.5 Chr10:100987542-100994403
No rat ortholog			OR6F1 NM_001005286.1 Chr1:247711828-247712755
No rat ortholog			OR10A5 NM_178168.1 Chr11:684568-6846636
No rat ortholog			H2AB3 NM_080720.1 ChrX:155459418-155459935
No rat ortholog			OR14A2 NM_001355292.1 Chr1:247722956-247748158
No rat ortholog			CYP2C9 NM_000771.4 Chr10:94938657-94990091
No rat ortholog			CYP2C8 NM_000770.3 Chr10:95036771-95069497
No rat ortholog			CYP4A11 NM_000778.4 Chr1:46929187-46941476
No rat ortholog			BCL2A1 NM_004049.4 Chr15:79960891-79971196
No rat ortholog			ZSCAN5A NM_001322072.1 Chr19:56221302-56368383

Table 15. Genome Positions of Rat *Mcs3* Non-Coding DNA and Orthologous Human Genome Segments

lncRNA/miRNA	Accession number	Genomic Position	Species*
LOC102556513	XR_590364.1	Chr1:143846546-143861632	Rat
LOC103691175	XR_590365.1	Chr1:143930927-143936603	Rat
LOC108348871	XR_001835889.1	Chr1:144778613-144798063	Rat
LOC103691179	XR_590366.2	Chr1:145146668-145149394	Rat
LOC103691180	XR_590367.2	Chr1:145360463-145363189	Rat
LOC102552825	XR_350537.1	Chr1:145448880-145450795	Rat
LOC103691181	XR_590368.1	Chr1:145505885-145521853	Rat
LOC108349601	XR_001835891.1	Chr1:145525669-145537559	Rat
LOC108349171	XR_001835892.1	Chr1:146289822-146320389	Rat
LOC108349102	XR_001835893.1	Chr1:146346478-146376238	Rat
LOC102553298	XR_590369.2	Chr1:146682691-146706084	Rat
LOC108349602	XR_001835899.1	Chr1:146898565-146906850	Rat
LOC690082	XM_017590171.1	Chr1:146966353-146972459	Rat
LOC100911002	XM_003748903.4	Chr1:146977349-146989856	Rat
LOC100911047	XM_006229628.3	Chr1:147000036-147012436	Rat
LOC100366231	XM_003748905.4	Chr1:147060790-147072122	Rat
LOC100911148	XM_017590168.1	Chr1:147077010-147083118	Rat
LOC100361434	XM_017590456.1	Chr1:147524188-147596924	Rat
LOC102550489	XR_590372.2	Chr1:147641561-147642719	Rat
LOC100911718	XM_003748910.4	Chr1:147883945-147887671	Rat
LOC103691183	XR_590373.2	Chr1:148193185-148196949	Rat
LOC100361547	XM_008759635.1	Chr1:148232437-148287458	Rat
LOC100911879	XR_590374.2	Chr1:148363574-148380117	Rat
LOC102546990	XM_006229649.3	Chr1:148445119-148446339	Rat
LOC100912097	XM_003748913.1	Chr1:148862492-148863446	Rat
LOC679574	XM_008759645.1	Chr1:149437495-149476747	Rat
LOC100909966	XM_003748916.2	Chr1:149602345-149603284	Rat
LOC100910049	XM_017590559.1	Chr1:149632087-149633029	Rat
LOC100909935	XM_003748915.3	Chr1:149777725-149778838	Rat
LOC100910009	XM_003748917.2	Chr1:149858459-149859555	Rat
LINC00927	NR_033833.1	Chr15:80263067-80341805	Human
LOC101929560	XR_932523.2	Chr15:80344848-80362464	Human
LOC101929586	NR_120363.1	Chr15:80554608-80562944	Human
LOC107984749	XR_001751630.2	Chr15:80896190-80911991	Human
LOC102723985	XR_001751634.1	Chr15:81614941-81664130	Human
LOC105370921	XR_932526.2	Chr15:81665145-81690642	Human
LOC102724001	XR_932527.2	Chr15:81912868-81967182	Human
LOC105370922	XR_932529.2	Chr15:81977933-82013733	Human
LOC105370923	XR_932531.2	Chr15:82026116-82032619	Human
LINC01583	NR_120367.1	Chr15:82088593-82097694	Human
LOC105370924	XR_932532.1	Chr15:82096581-82099672	Human
LOC105370932	XR_932541.2	Chr15:83283474-83318663	Human
LOC105370933	XR_932544.1	Chr15:83345641-83350586	Human
LOC105370934	XR_932545.2	Chr15:83365867-83368716	Human
LOC105370935	XR_001751646.1	Chr15:83944102-83949251	Human
LOC112268243	XR_002958412.1	Chr19:56314620-56341105	Human
MIR5572	NR_049837.1	Chr15:80581102-80581239	Human
MIR549A	NR_030393.1	Chr15:80841977-80842073	Human
MIR4514	NR_039739.1	Chr15:80997416-80997473	Human
MIR1184-3	NR_036260.1	ChrX:155457516-155457615	Human

* *Rattus norvegicus* assembly RGSC 6.0/Rn6, Human assembly GRCh38/hg38

percentage, and fat body mass. A lollipop diagram of GWAS variants contained within these two human syntenic regions is shown in Figure 20. Only one of these variants has a direct association with breast cancer. The remaining variants stem from breast cancer associated traits, namely body mass.

Discussion:

The focus of this study was to enhance our understanding of genetic risk and predisposition of human breast cancer using a rat mammary cancer model. Specifically, I sought to further narrow *Mcs3* to reduce the pool of potential candidate breast cancer susceptibility genes. In this way, a subset of genomic variants and genes can be identified for orthologous risk analysis in human disease, and this rat model further utilized to investigate these genes and their underlying molecular mechanisms in breast/mammary carcinogenesis.

Mcs3 was first identified as a putative QTL associated with a decrease in the number of mammary tumors in female rats carrying the COP allele by Michael Gould's group. These studies conducted genome scans for loci that modulate induction of mammary carcinomas in (WFxCOP) F_1 x WF backcross rats [314]. Peak logarithm of odds (LOD) scores estimated an *Mcs3* interval of around 30 cM and predicted this loci contained potential mammary cancer resistance genes [314]. *Mcs3* was physically confirmed in 2017 using WF.COP congenic rat strains and delimited to a 29.4 Mb region on rat chromosome 1:143700228–171517317 (build 6.0/rn6) [243].

My congenic rat lines further delimit *Mcs3* to a 7.2 Mb region of rat chromosome 1 that spans from *rs8149408* to *rs107402736* (Chr1:143700228-150929594). I was

Table 16. Human Cancer-associated Genes Located in Rat *Mcs3*

Gene	Cancer association	Basis for Association
MPP1*	Lung adenocarcinomas and neoplasms [327]	Meta-analysis of microarray datasets
IL16	cutaneous T cell lymphoma [328], multiple myeloma [329], breast [330, 331], gastrointestinal [331, 332], colorectal [332], renal cell carcinoma [333], lung [334], prostate [335, 336]	Flow cytometry [328, 331]. ELISA of patient serum samples [329, 331-333], Cell culture tumor spheroids and tumor xenografts [330], RT-qPCR based on patient genotypes [334], GWAS [335, 336]
FOLH1 (PSMA)	Prostate [337, 338], breast [339, 340]	RT-PCR and PDX models from patient samples [337], IHC of patient tissue microarrays [338], IHC and IF of patient samples [339, 340]
NOX4	Glioblastoma [341], thyroid [342], melanoma [343], gastric [344], colorectal [345], prostate [346], lung [347], endometrial [348]	RT-PCR and IHC of patient samples [341-347], tumorigenicity in nude mice [343], expression microarray [344-347], xenografts [347], <i>in situ</i> hybridization [348]
BCL2A1**	Breast [349, 350]	IHC of patient samples [349], differential expression analysis [350]
CYP2C19/ Cyp2c6v1	Breast [351, 352]	Global screening array of patient genotypes [351], genotyping of patient samples [352]
EFL1	Glioma [353], breast [354]	WB, IHC of patient samples, xenograft tumor model [353], qRT-PCR [353, 354], RNA-seq [354]
CYP2C8/ Cyp2c79	Breast [355], hepatocellular carcinoma [356]	genotyping of patient samples [355], RT-PCR and IHC of patient samples [356]
CYP4A11	Hepatocellular carcinoma [357, 358], renal cell carcinoma [359]	co-expression network from clinical data, RNA-seq [357], IHC, WB, RT-PCR of patient samples [358, 359]
ARNT2	Hepatocellular carcinoma [360], oral squamous cell carcinoma [361], breast [362], lung [363], gastric [364]	IHC and tissue microarray of patient samples [360], qRT-PCR, WB, and IHC of patient samples [361], whole-genome microarray [362], xenograft tumors [363], IHC of patient samples [363, 364]
CEMIP	Pancreatic [365], colorectal [366, 367], gastric [368, 369], breast [370], ovarian [371], prostate [372]	ELISA of patient serum [365], microarray analysis [366, 372], transcript profiling [367], IHC of patient samples [367, 368, 371, 372], RT-PCR [368,

		369], cDNA microarray [369], IHC of tissue microarray [370]
ABHD17C	Breast [373]	gene expression profiles from PDX
TLNRD1 (MESDC1)	Hepatocellular carcinoma [374]	RT-PCR of patient samples, tumor xenografts
MEX3B	Melanoma [375]	RNA-seq and RT-qPCR of patient samples
TM6SF1	Breast [376], hepatocellular carcinoma [377]	Methylation analysis in patient samples [376, 377]
BNC1	Pancreatic [378], breast [379], hepatocellular carcinoma [380], squamous cell carcinoma [381, 382]	Methylation analysis in patient samples [378-380], RT-PCR of patient samples [380-382], expression array and IHC from patient samples [382]
SH3GL3	Glioblastoma [383], colorectal [384], oral squamous cell carcinoma [385]	Expression microarray [383, 385], IHC from patient samples [383], methylation analysis in patient samples [384], qRT-PCR [385]
ADAMTSL3	Colorectal [386], hepatocellular carcinoma [387]	<i>in situ</i> hybridization and qRT-PCR of patient samples [386], IHC of patient samples [386, 387], <i>in vivo</i> screening [387]
LINC01583	Colorectal [388]	Expression profile screening

*Not orthologous to rat *Mcs3*

**Rat segment not located at *Mcs3*

Table 17. Breast Cancer Risk Correlated Traits with Human GWAS Significant Variants in Genomic Segments Orthologous to Rat *Mcs3*

Variant dbSNP ID	P-Value*	Trait	Genomic position	Gene/locus	Accession number
rs7165759	1 x 10 ⁻¹⁰	visceral adipose tissue measurement	Chr15:80696259	ABHD17C	NM_021214.2
rs12914623	2 x 10 ⁻¹⁶	body mass index	Chr15:80701229	ABHD17C	NM_021214.2
rs57783949	4 x 10 ⁻⁸	body mass index	Chr15:80710515	ABHD17C	NM_021214.2
rs34769775	9 x 10 ⁻¹⁴	body mass index	Chr15:80696831	ABHD17C	NM_021214.2
rs2759315	5 x 10 ⁻¹¹	body mass index	Chr15:80717305	ABHD17C	NM_021214.2
rs12593036	2 x 10 ⁻¹⁵	body mass index	Chr15:80766311	ABHD17C	NM_021214.2
rs12593088	5 x 10 ⁻¹²	Waist-hip ratio	Chr15:80766299	ABHD17C	NM_021214.2
rs149479659	2 x 10 ⁻⁶	Breast cancer specific mortality in estrogen receptor positive breast cancer	Chr15:80763524	ABHD17C	NM_021214.2
rs530826342	3 x 10 ⁻⁶	Fat body mass	Chr15:81398425	TMC3	NM_001080532.3
rs12324805	7 x 10 ⁻⁶	body mass index	Chr15:82059859	MEX3B	NM_032246.6
rs111491984	1 x 10 ⁻⁹	age at menarche	Chr15:82195898	EFL1	NM_024580.6
rs11325	5 x 10 ⁻⁹	Body height	Chr15:81308999	IL16	NM_004513.6
rs4448892	2 x 10 ⁻¹²	Body height	Chr15:81196722	IL16	NM_004513.6
rs17875533	1 x 10 ⁻¹⁸	Body height	Chr15:81305884	IL16	NM_004513.6
rs11857976	2 x 10 ⁻³⁰	Body height	Chr15:81531197	AC060809	AC060809.1
rs28463783	1 x 10 ⁻¹⁰	Body height	Chr15:81954237	AC104041	AC104041.1
rs2654210	6 x 10 ⁻⁸	Waist-hip ratio	Chr15:82113300	LINC01583	NR_120367.1
rs35056343	2 x 10 ⁻¹³	Body height	Chr15:81543120	AC060809	AC060809.1
rs2554380	9 x 10 ⁻⁷	Body height	Chr15:83647132	ADAMTSL3/ SH3GL3	NM_207517.3/ NM_003027.5
rs10906982	2 x 10 ⁻⁸	Body height	Chr15:83899406	ADAMTSL3	NM_207517.3
rs11259933	1 x 10 ⁻¹⁹	Body height	Chr15:83911404	ADAMTSL3	NM_207517.3
rs7183263	4 x 10 ⁻⁷	Body height	Chr15:83904289	ADAMTSL3	NM_207517.3
rs1383484	9 x 10 ⁻⁷	Body height	Chr15:83854003	ADAMTSL3	NM_207517.3
rs11259936	2 x 10 ⁻³⁵	Body height	Chr15:83911830	ADAMTSL3	NM_207517.3
rs2401171	4 x 10 ⁻²¹	Body height	Chr15:83888924	ADAMTSL3	NM_207517.3
rs2257011	1 x 10 ⁻⁴⁷	Body height	Chr15:83597393	SH3GL3	NM_003027.5
rs7162542	8 x 10 ⁻⁵⁵	body height, BMI-adjusted waist circumference, BMI-adjusted hip circumference	Chr15:83845538	ADAMTSL3	NM_207517.3
rs4842924	7 x 10 ⁻¹⁰	Lean body mass	Chr15:83918855	ADAMTSL3	NM_207517.3
rs2585061	5 x 10 ⁻¹³	Body height	Chr15:83678999	ADAMTSL3	NM_207517.3
rs1896796	4 x 10 ⁻⁶	Body height	Chr15:83606228	SH3GL3	NM_003027.5
rs8024628	5 x 10 ⁻¹⁹	body height, BMI-adjusted waist circumference, BMI-adjusted hip circumference	Chr15:83917711	ADAMTSL3	NM_207517.3
rs950169	2 x 10 ⁻⁷	Body height	Chr15:84037709	ADAMTSL3	NM_207517.3
rs2562784	2 x 10 ⁻⁶	Body height	Chr15:83617740	SH3GL3	NM_003027.5
rs28728297	7 x 10 ⁻⁶	Body height	Chr15:83877660	ADAMTSL3	NM_207517.3
rs2562781	2 x 10 ⁻⁹	BMI-adjusted hip circumference	Chr15:83643816	ADAMTSL3/ SH3GL3	NM_207517.3/ NM_003027.5
rs4842838	6 x 10 ⁻²⁷	BMI-adjusted waist circumference, body height	Chr15:83913372	ADAMTSL3	NM_207517.3
rs8030379	9 x 10 ⁻²⁷	BMI-adjusted waist circumference	Chr15:83922158	ADAMTSL3	NM_207517.3
rs2030839	1 x 10 ⁻¹⁵	BMI-adjusted waist circumference	Chr15:83915207	ADAMTSL3	NM_207517.3

rs111470917	7 x 10 ⁻³⁷	Body height	Chr15:83916787	ADAMTSL3	NM_207517.3
rs8038454	4 x 10 ⁻¹¹	Body height	Chr15:83670684	ADAMTSL3	NM_207517.3
rs11856122	2 x 10 ⁻³¹	Body fat distribution	Chr15:83907596	ADAMTSL3	NM_207517.3
rs8031704	1 x 10 ⁻¹⁵	Body fat percentage	Chr15:83982433	ADAMTSL3	NM_207517.3
rs62025778	1 x 10 ⁻²⁰	BMI-adjusted waist circumference	Chr15:83776366	ADAMTSL3	NM_207517.3
rs75821416	2 x 10 ⁻¹³	BMI-adjusted waist circumference	Chr15:83657008	ADAMTSL3	NM_207517.3
rs75666428	8 x 10 ⁻¹¹	BMI-adjusted waist circumference	Chr15:84012650	ADAMTSL3	NM_207517.3
rs55685171	6 x 10 ⁻¹⁰	BMI-adjusted waist circumference	Chr15:83757828	ADAMTSL3	NM_207517.3
rs7169595	9 x 10 ⁻⁹	BMI-adjusted waist circumference	Chr15:83736822	ADAMTSL3	NM_207517.3
rs140574883	1 x 10 ⁻¹³	BMI-adjusted waist circumference	Chr15:84074328	AC027807	AC027807.1
rs141724470	6 x 10 ⁻⁹	BMI-adjusted waist circumference	Chr15:84044702	ADAMTSL3	NM_207517.3
rs11259926	7 x 10 ⁻²¹	BMI-adjusted waist circumference	Chr15:83897496	ADAMTSL3	NM_207517.3
rs4842918	1 x 10 ⁻⁶²	BMI-adjusted waist circumference	Chr15:83868247	ADAMTSL3	NM_207517.3
rs4583201	5 x 10 ⁻¹¹	BMI-adjusted waist circumference	Chr15:83820297	ADAMTSL3	NM_207517.3
rs113817743	4 x 10 ⁻⁹	BMI-adjusted waist circumference	Chr15:83817917	ADAMTSL3	NM_207517.3
rs4842926	1 x 10 ⁻⁴⁴	BMI-adjusted waist circumference	Chr15:83939736	ADAMTSL3	NM_207517.3
rs8035886	2 x 10 ⁻³⁴	BMI-adjusted waist circumference	Chr15:83790608	ADAMTSL3	NM_207517.3
rs77378263	3 x 10 ⁻¹⁴	BMI-adjusted waist circumference	Chr15:83571369	SH3GL3	NM_003027.5
rs2730075	6 x 10 ⁻¹²	BMI-adjusted waist circumference	Chr15:83645689	ADAMTSL3/ SH3GL3	NM_207517.3/ NM_003027.5
rs75340848	2 x 10 ⁻¹⁰	BMI-adjusted waist circumference	Chr15:83420223	AC103876	AC103876.1
rs2220856	5 x 10 ⁻²²	BMI-adjusted waist circumference	Chr15:83526475	SH3GL3	NM_003027.5
rs10152514	5 x 10 ⁻¹⁴	BMI-adjusted waist circumference	Chr15:83583309	SH3GL3	NM_003027.5
rs2732151	8 x 10 ⁻¹⁰	BMI-adjusted waist circumference	Chr15:83534933	SH3GL3	NM_003027.5
rs4843136	2 x 10 ⁻⁸	BMI-adjusted waist circumference	Chr15:83403098	AC103876	AC103876.1
rs4842921	8 x 10 ⁻¹¹	BMI-adjusted wait to hip ratio	Chr15:83887871	ADAMTSL3	NM_207517.3
rs185392832	2 x 10 ⁻⁸	Body height	Chr15:83162219	HDGFL3	NM_016073.4
rs6602982	3 x 10 ⁻⁴²	Body height	Chr15:83588992	SH3GL3	NM_003027.5
rs118136305	2 x 10 ⁻¹⁶	Body height	Chr15:83769541	ADAMTSL3	NM_207517.3
rs2135880	3 x 10 ⁻²²²	Body height	Chr15:83920861	ADAMTSL3	NM_207517.3
rs11630762	5 x 10 ⁻¹⁰	Waist to hip ratio	Chr15:83946227	ADAMTSL3	NM_207517.3
rs2289125	2 x 10 ⁻¹⁶	Body height	Chr11:89491285	NOX4	NM_016931.5
rs317162	5 x 10 ⁻⁹	body mass index	Chr11:89371405	NOX4	NM_016931.5
rs61886764	3 x 10 ⁻⁸	BMI-adjusted waist circumference	Chr10:94912317	CYP2C9	NM_000771.4
rs530826342	3 x 10 ⁻⁶	fat body mass	Chr15:81398425	TMC3-AS1	NR_120365.1

*P-value for association

** Homo sapiens assembly GRCh38/hg38

Figure 20: Diagram of overlapping GWAS breast cancer associated traits orthologous to rat *Mcs3*. Shown are human syntenic regions at chr15:80005820-82285404 and chr15:83134545-84130720. Each color represents a different trait as indicated by the legend. Multiple nearby variants associated with the same trait share a circle.

unable to uncover additional informative markers between WF and COP strains to further fine map *Mcs3*, largely due to the published genomes on the Rat Genome Database. The uploaded COP genome has a high depth of coverage and number of reads, but the other rat strain genomes searched possessed a low depth of sequencing across reported SNPs. My inability to find informative SNPs was likely due to the uploaded sequencing data that were not high quality enough to identify true variants differing from the COP genome. This genome possessed a high frequency of variants because it is highly dissimilar from the Brown Norway rat reference genome, but likely is more similar to the Wistar genomes than the low-quality genome sequencing makes it appear. This difficulty in finding informative markers at higher resolution in fine mapping *Mcs3* suggests WF and COP share similar haplotypes at *Mcs3*. Thus, more work in finding genetic markers will need to be conducted if future work in deriving more congenic lines is pursued. *Mcs3* is also at a shortened enough interval now that it could also possibly be sequenced between WF and COP strains to identify and evaluate causal genetic elements or transcriptional differences that contribute to mammary cancer susceptibility.

A single COP allele was predicted to reduce mammary carcinoma susceptibility by 42% in female rats heterozygous at the original *Mcs3* locus [164]. This study found that female rats homozygous for the COP allele at the delimited *Mcs3* locus had a 57% reduction in mammary cancer susceptibility. The difference in the susceptibility between the two studies might be explained by either a possible degree of dominance of the COP allele at the *Mcs3* locus, or by background COP alleles potentially present in the females included in the original linkage analysis.

Breast carcinomas are usually classified based on histological appearance, with up to 21 histological types identified via differing cytoarchitectural features and

morphological variation [389]. Invasive ductal carcinomas (IDC), specifically, account for 80% of diagnosed female breast cancers [390]. The majority of IDCs do not exhibit enough histological features, other than proliferative invasion through the ductal basement membrane, to be classified as a specific tumor subtype [389, 391]. These IDCs of *no special type*, or *not otherwise specified* account for 75% of invasive carcinomas [391]. It is also well established that chemically-induced rat mammary tumors share similar histopathology to human breast carcinomas [221, 392, 393]. In pubescent nulliparous rats, carcinogenic initiation using 7,12-dimethylbenz(a)anthracene (DMBA) primarily occurs in the terminal end buds and terminal ducts within mammary tissue, giving rise to adenocarcinomas of ductal cell origin [394]. These are intraductal and invasive ductal carcinomas, similar to what is observed in the majority of human breast malignancies of *no specific type* [394]. The majority of DMBA-induced rat mammary carcinomas are classified as invasive papillary carcinomas, comprising 32% of tumor histology [394]. The invasive cribriform carcinoma is the second most common histologically-defined rat mammary carcinoma at 27% of DMBA-rat mammary cancers [394]. These mammary IDC subtypes were the most frequently observed tumor histologies in the WF strain and each *Mcs3* WF.COP congenic lines H, I, J, and K.

The papillary carcinoma is defined by branching fibrovascular cores throughout the lesion that contain variable degrees of stromal fibrosis (Fig 14, Arrow A) [395-397]. These cores support neoplastic epithelial proliferations, which form papillary projections that comprise this invasive growth pattern [394-397] (Fig 14, Arrow B). The invasive cribriform carcinoma is defined by invasion of the stroma by neoplastic epithelial cells that form nestlike tumor structures [398, 399]. These tumor nests are characterized by glandular

spaces with no solid component that create a sieve-like appearance (Fig 15, Arrow A) [394, 398, 399]. The cribriform architecture also displays a surrounding desmoplastic stroma with lymphocyte infiltration (Fig 15, Arrow B) [394, 399]. Since these two most common DMBA-mammary tumor subtypes were the majority histologies observed in each of the *Mcs3* WF.COP congenic lines, it was concluded that these rat lines share the same histopathological tumor development pathways in response to DMBA-initiation. As a result, it wasn't the tumor subtype or pathology that accounted for the differences in tumor multiplicity between congenic lines. This also indicates that the mammary tumor resistant phenotype observed in line J, as opposed to the susceptible phenotypes of lines H, I, and K, do not result in differences in tumor development and morphology.

In addition to mammary cancer resistance, a secondary phenotype of lower body mass was documented in line J rats. This indicates a developmental delay in line J growth; however, it doesn't appear to affect mammary gland development. H&E staining showed the observed morphologies match known rat mammary gland developmental structures, such as terminal end bud appearances during puberty, and appears consistent between line J and WF females across all developmental timepoints. Lower body mass also doesn't seem to be associated with a developmental delay in body growth as it persists over the lifetime of line J rats.

It is possible that these pleiotropic traits of mammary cancer resistance and low body mass phenotypes are connected, as obesity, characterized by excess body fat, is associated with greater breast cancer incidence [400-402]. Specifically, obesity is associated with larger tumor size, positive lymph node status, shorter time to disease recurrence, and mortality [401, 403-405]. There exists a positive association between

obesity and breast cancer risk in postmenopausal women [406-409], likely due to adipose tissue being the main site of estrogen synthesis in postmenopausal women [410] and higher concentrations of estrogen in the excess fat reserves of obese women [411]. These greater estrogen levels could be expected to drive estrogen-dependent tumors, which is supported by the fact that most postmenopausal breast cancer cases in women with obesity are ER-positive [412-416]. Inversely, there is a negative relationship between obesity and breast cancer risk in premenopausal women [411, 417-419]. It has been speculated this may be due to less estrogen in circulation during premenopausal cycling [409], and suggested that this effect is reversed after adjusting for breast density [420-422]. Extra adipose tissue in general mimics an inflammatory transcriptome and produces inflammatory cytokines, creating a microenvironment conducive to cancer initiation, invasion, and metastasis [423-425]. The higher body mass in the WF strain as compared to line J could result in subclinical inflammation of mammary tissue that contributes to the differences in mammary tumor incidence. Conversely, the lower body mass observed in line J could lend a protective effect to disease development, similar to what is observed in human epidemiological studies.

The lower body mass phenotype in line J rats appears to be a sex-dependent trait. In general housing, only line J males had a significantly lower body mass compared to WF. Although the mammary tumor phenotype is only studied in females, this body mass phenotype indicates that the *Mcs3* genotype also has an effect in males. In female rats, it wasn't until placed in a female-only environment that female line J rats displayed a lower body mass compared to WF. It appears that the presence of male rats somehow masks the effect of *Mcs3* genotype on female body mass. The only apparent environmental difference

is the lack of male pheromones. In rodents, male pheromones can induce a physiological change in the female endocrine and reproductive system [426]. In rats, this includes induction of puberty, a reduction in the duration of the estrus cycle, induced diestrus staging, and restoration of estrus cycling in aging females [426-428]. These endocrine disruptions are due to changes in hormonal signaling. For example, male urinary pheromones have been demonstrated to increase plasma progesterone levels in females [429]. The endocrine system also has a role in body weight regulation, disruptions to which could be theorized to have an effect on overall body mass. The genomic actions of ER α specifically has a known downstream effect on body weight [430, 431]. Estrogen is demonstrated to reduce food intake and body weight in ovariectomized rats, with restoration of eating behavior and normal body weight restored with estradiol treatment [431, 432]. Targeted deletion of an ER α subunit was also reported to increase body mass and adiposity [433]. Additionally, estrogen modulates leptin synthesis and secretion, and so regulates energy homeostasis and body weight in an indirect manner [434-436]. Thus, the differences in female line J body mass with and without the presence of male pheromones could be due to disruptions in endocrine signaling. The lower body mass as compared to WF females is presumed to be an effect of the genomic interplay of the *Mcs3* reduced mammary cancer susceptibility allele.

Rat *Mcs3* was found to align to three human syntenic loci on chromosomes 11 and 15. These rat and human orthologous regions contain 16 annotated genes with cancer associations, as well as an additional rat only gene, and human only gene and lncRNA, not annotated in either orthologous regions. Of these genes, 11 have reported associations with breast cancer. There is reported significant increases in the cytokine *interleukin-16 (IL16)*

in serum levels of stage III and IV breast cancer patients without or after chemo/radiotherapy compared to healthy controls [331]. Elevated *IL16* levels in tumor spheroids and xenografts also result in greater migration and invasion of blood monocytes and macrophages in the tumor microenvironment, potentially serving as a chemoattractant in tumorigenesis [330]. *Folate hydrolase 1 (FOLH1)*, also known by its protein product prostate-specific membrane antigen (PSMA), has been shown to localize to tumor cells of primary breast tumors and neovasculature of breast tumors and distant metastasis, serving as a potential therapeutic vascular target [339]. Additionally, the highest levels of PSMA are observed in the aggressive triple negative breast cancer molecular subtype [340]. The pro-survival/apoptotic-inhibitor *B-cell lymphoma 2-related protein A1 (BCL2A1)* is upregulated in breast tumor samples as compared to non-diseased, where it potentially has a role in protecting pro-inflammatory cells from apoptosis [350, 437]. Increased expression is positively associated with advanced TNM stage and poor prognosis in triple negative breast cancer [349]. Overexpression of *BCL2A1* has also been demonstrated to prevent mammary gland involution via apoptosis, indicating a physiological role in mammary gland function [438]. Inherited mutations of *cytochrome P450 2C19 (CYP2C19)*, which has a major function in estrogen catabolism, have poor associations in breast cancer patients, including triple negative breast cancer [351, 352]. CYP2C19 may then serve as a potential prognostic biomarker. Additionally, gene variants of *cytochrome P450 2C8 (CYP2C8)* are associated with increased risk of breast cancer [355]. *Elongation factor like GTPase 1 (EFL1)* is a domain of *elongation factor-1 alpha (EF1 alpha)*, which has a role in proliferation, metastasis, and apoptosis [353, 439, 440]. Vitamin D3 has been shown to directly upregulate expression of *EFL1* in *ex vivo* explants of human breast tissue where it

may serve in a potential tumor suppressor role [354]. *Aryl hydrocarbon receptor nuclear translocator 2 (ARNT2)* is significantly overexpressed in breast tumors as compared to normal breast tissue, and positively correlates with patient relapse-free survival and overall survival [362]. As a result, it may be useful as a prognostic biomarker of breast cancer. *Cell migration-inducing and hyaluronan-binding protein (CEMIP)*, formerly known as *KIAA1199*, has significantly increased expression in invasive breast carcinomas as compared to non-diseased breast tissue, and expression is inversely associated with patient survival [370]. CEMIP overexpression *in vitro* has also been demonstrated to increase migration of breast cancer cells and markers of epithelial-to-mesenchymal transition, as well as mammary gland tumor metastasis *in vivo*, serving a critical role in producing a metastatic cancer phenotype [370]. High expression of *alpha/beta hydrolase domain-containing protein 17C (ABHD17C)* has been reported in breast cancer patient samples and patient-derived tumor xenografts, although no studies into the molecular role in breast cancer have been done to date [373]. The promoter of *transmembrane 6 superfamily member 1 (TM6SF1)* was found to be significantly differentially methylated in normal breast tissue as compared malignant breast tissue. Though the function of TM6SF1 remains unknown, it may serve as a potential methylation biomarker for early breast cancer detection [376]. The transcription factor *zinc finger protein basonuclin-1(BNC1)* is reported to be methylated and silenced during breast to brain metastasis, but not in primary breast carcinomas, suggesting a role in tumor progression and metastasis [379].

The majority of GWAS-nominated variants identified in human orthologous syntenic regions to rat *Mcs3* reside in *SH3 Domain Containing GRB2 Like 3, Endophilin A3 (SH3GL3)* and *ADAMTS-like protein 3 (ADAMTSL3)*. These variants are associated

with body mass and ratio traits, including body mass index, waist-hip ratio, fat body mass, body height, lean body mass, BMI-adjusted hip circumference, BMI-adjusted waist circumference, body fat distribution, body fat percentage, BMI-adjusted waist circumference, and fat body mass. These regions contain no direct association with breast cancer; however, are relevant to our congenic studies as line J was positive for the *Mcs3* reduced susceptibility locus and displayed a significantly lower body mass as compared to the other congenic lines. This suggests that *Mcs3* is indeed modulating both body mass and mammary cancer susceptibility, and that these orthologous genic regions in the human that are associated with body mass may have a novel, secondary role in cancer development. Additionally, there was one variant, *rs149479659*, with a direct breast cancer association, which indicates that this genetic region may warrant ultra-fine mapping to further investigate underlying susceptibility mechanisms at this locus. Commonly in these susceptibility loci, it is gene overexpression that has a mechanistic role in driving a cancer phenotype. However, genetic variants, especially in non-coding regions, modulate gene expression levels by altering the function of regulatory DNA. Thus, unannotated variants within rat *Mcs3* and human orthologous regions may alter the expression patterns of nominated candidate cancer genes contained in these loci. Future studies aimed at further physical mapping of *Mcs3* would reduce the pool of candidate genes and create a smaller orthologous region in the human genome in which to fine map and identify causal disease variants. Since WF and COP seem to share similar haplotypes at *Mcs3*, it may also be more feasible to directly sequence this locus in both strains and isolate potential causal differences leading to altered susceptibility.

CHAPTER IV

A METHOD TO PRE-SCREEN RAT MAMMARY GLAND WHOLE-MOUNTS
PRIOR TO RNASCOPE

Introduction:

Protein, DNA, and RNA biomarkers are important clinical diagnostic and prognostic tools [442, 443]. Gene expression profiling has highlighted an abundance of novel RNA biomarkers associated with cancer [443-445]. Expression profiling of specific RNAs using whole tissue homogenates is problematic because of signal interference from multiple cell types. Furthermore, spatial and cell-specific information are lost in approaches using disrupted tissue [443, 446]. New technologies, including single-cell sequencing and RNAscope, have made single-cell RNA transcript profiling a reality. Gall and Pardue introduced molecular hybridization using radiolabeled nucleic acid sequences *in situ* [447, 448]. Since their work, many additional techniques have been established, including RNAscope, a proprietary RNA *in situ* hybridization (RNA ISH) method that provides quantitative gene expression analysis within intact tissue [446].

Commercially available RNAscope technology utilizes a novel target probe design, along with a series of hybridization-based signal amplification steps, to provide single molecule visualization within specific cell populations of intact tissue. Oligo “Z probes”

Adapted from Duderstadt et al. (2021). [441] See Appendix

for RNAscope contain 18-25 base pair complementarity to a target transcript, a spacer sequence, and a 14 base pair tail sequence. These assays require adjacent target probes (a double Z pair) to hybridize contiguously to target RNA. The combined tail sequences of a double Z pair provide for hybridization of a preamplification scaffold, which contains multiple binding sites for subsequent amplifier molecules. Next, fluorescent-dye or chromogenic labeled probes bind to the amplifier molecules, which contain multiple binding sites for the labeled probes. Double Z probe pairing results in target-specific amplification and a reduction in background signal because binding of a single probe is not sufficient for preamplification scaffold hybridization [446].

Rodent models have been used extensively to study morphological changes during mammary gland development and disease progression [449-451]. Gross morphology of the mammary gland can be examined by whole-mounting tissue to a microscope slide and staining of intact mammary gland tissue [450, 451]. Alum carmine is a biological nuclear stain derived from dried female cochineal insects [452]. Mammary ductal structures are easily visualized when fat-cleared mammary gland tissue is stained with carmine [449]. Whole-mounts are extremely useful to observe epithelial changes that occur during mammary gland development, function, and disease. Tissue morphologies can be excised, processed, and sectioned for further evaluation. This approach provides efficiency in capturing disease morphologies, as standard procedures would require extensive serial sectioning to find morphologies of interest [451]. For example, rat mammary carcinogenesis serves to model the natural histopathological lesions and malignancies found in human breast disease. Visualization of rat mammary gland ductal structures *in situ* is useful because, similar to a majority of human breast cancers, neoplasms arise in

mammary ducts [391, 453, 454]. Screening tissue whole-mounts for morphologies of interest is a valuable technique to visualize and study changes occurring during carcinogenesis [453, 455].

RNAscope assays are designed and recommended by the manufacturer to be used on recently processed formalin fixed and paraffin embedded (FFPE) or cryopreserved tissue samples. Morphological features are retained in FFPE tissues, making it a preservation method of choice for decades. Integrity of RNA is preserved for long periods of time in FFPE tissues [456, 457]. This is important because nucleic acid degradation is a pervasive problem that must be avoided in samples used for any RNA ISH method, including RNAscope. Loss of nucleic acid quality has a negative impact on the reproducibility and quantitative accuracy of subsequent assays. The duration of fixation and pre-treatment conditions can negatively impact nucleic acid integrity and compromise RNAscope assays [458, 459]; thus, care must be taken to limit exposure time to harsh conditions prior to paraffin embedding tissue. We report that RNAscope can be effectively utilized following screening of mammary gland whole-mounts for lesions of interest. This is useful as it allows for the identification and study of diseased tissue morphologies without apparent loss of RNA integrity. We show, using probes targeting genes with known expression level differences, that the quantitative capacity of RNAscope is retained in tissue excised from mammary gland whole-mounts. This is applicable to mammary gland biology studies requiring pre-screening of mammary gland whole-mounts.

Protocol:***Animal husbandry***

Inbred Wistar Furth (WF/Hsd; Envigo) female rats were maintained in an AAALAC-approved facility on a 12 h light/dark cycle and provided LabDiet 5001 Rodent Diet (PMI® Nutrition International) and water *ad libitum*. The University of Louisville Institutional Animal Care and Use Committee approved all animal protocols.

DMBA Administration and Mammary Gland Tissue Resection

At 50-55 days of age, 7,12-dimethylbenz(a)anthracene (DMBA Acros Organics, CAS#: 57-97-6, 20 mg/mL in sesame oil) was given by single oral gavage (65 mg DMBA/kg body mass) to induce mammary carcinogenesis. Rats were euthanized by CO₂ asphyxiation at four or twelve weeks following DMBA administration and mammary glands excised. Briefly, one skin incision was made at the lower abdomen and extended forward, along the midline, to the neck (Fig 21). Four additional skin incisions, extending down each limb from the midline, were made and skin was separated from the body cavity to reveal mammary glands. Abdominal 4th mammary glands (Fig 21) were used in this study. Mammary gland tissue was carefully trimmed away from skin by moving dorsally. Figure 22 contains a flowchart of tissue processing procedures used in this study. One mammary gland from each rat was immediately processed by FFPE, while the other was processed as a whole-mount. Tissues were fixed overnight in 10% neutral buffered formalin.

Whole-Mount Preparation:

Following resection, mammary glands were immediately stretched across an electrostatically charged slide and fixed by submerging in 10% neutral buffered formalin at room temperature overnight. This whole-mount procedure minimized solvent exposure and staining time [455]. Slides were removed from formalin and placed into an acetone bath at room temperature overnight to de-fat. Overnight in acetone did not always completely clear the whole-mount of fat; however, it was sufficient time for successful carmine staining to visualize mammary gland structures and lesions.

Following defatting of mammary glands, slides were submerged in an alum carmine staining solution, prepared by boiling 1g of carmine and 2.5g of potassium alum in 500 mL of RNase-free DI water for 20 minutes. Carmine solution was stored at 4°C and reused after filtering to remove oil. Carmine staining took 1-2 days depending on mammary gland thickness. Glands were considered fully stained when white tissue was no longer visible through the microscope slide. Whole-mounts, not used for RNAscope analysis, were dehydrated in ethanol gradients, cleared overnight in xylene, and submerged in room temperature mineral oil for long-term storage.

Mammary gland tissue processing, embedding, and sectioning:

Morphological structures and lesions of interest, such as hyperplasia and ductal carcinoma *in situ* (DCIS) (Fig 23), within carmine stained whole-mounts were excised using a surgical scalpel. Excised tissue was placed in histology cassettes and processed using a standard tissue processor. Tissues were oriented with the flat side, originally against

Figure 21: Mammary gland tissue extraction. *Left panel:* to reveal and expose mammary gland tissue, an incision was made in the skin at the lower abdomen (black circle) and extended along the midline. Four additional incisions were made extending from the midline down each limb. Skin was separated from the body cavity to expose mammary glands. *Right panel:* Rats have six ventral mammary gland pairs, which are located in cervical, thoracic, abdominal, and inguinal regions.

Figure 22: Flow chart outlining difference in tissue preparation methods. Mammary glands were collected, processed, and used in RNAscope assays within 4 days (top). Alternatively, whole-mounts were processed and excised tissue used in RNAscope assays within 6 days of mammary gland collection (bottom). These whole-mounts had been processed and stained with alum carmine to pre-screen mammary glands for disease lesions.

the microscope slide, facing the bottom of the paraffin mold. Paraffin embedded tissues were stored at 4°C until sectioning. Tissues in paraffin blocks were sectioned one day *prior* to an RNAscope assay. This was important because RNA is more stable while the tissue is in paraffin blocks than after tissue sectioning [460]. Prior to sectioning, paraffin blocks were placed at -20°C for a minimum of 30 minutes to improve sectioning and reduce wrinkling of tissue sections. Tissues were cut into 4 µm sections and placed onto Superfrost Plus microscope slides (VWR). Other slide types, suitable for RNA ISH, may also be used. Up to three tissue sections, depending on tissue size, were placed on each slide. Slides were kept at room temperature overnight to completely dry.

Hematoxylin and Eosin (H&E) staining of sectioned mammary glands:

Slides were deparaffinized and rehydrated using three separate xylene washes, followed by single washes in 100% ethanol, 90% ethanol, 80% ethanol, and DI water. Tissues were stained with hematoxylin for three minutes, rinsed under running DI water for three minutes, soaked in bluing reagent for less than one minute, and rinsed with DI water. Slides were dipped at least three times in 100% ethanol to eliminate water before staining in eosin-Y for 30-45 seconds. Tissue samples were then dehydrated using washes of 90% ethanol, 100% ethanol, and 3 separate xylene washes, air dried, and coverslipped using permount or a similar mounting media.

RNAscope Assay

In this report, RNAscope assays were performed on abdominal 4th mammary gland pairs, one immediately processed by FFPE and the other whole-mounted, defatted, carmine

stained, and processed and paraffin embedded later. The RNAscope 2.5 HD Detection Reagent-RED kit (Advanced Cell Diagnostics #322360) was used according to manufacturer's protocol, except for the following modifications that served to minimize harsh pre-hybridization treatment conditions that could compromise fragile mammary tissue [461]. Slides were not rocked or agitated during any step of the hybridization procedure. Slides were deparaffinized in two xylene, followed by two 100% ethanol washes. A hydrophobic barrier was drawn around tissue sections with an ImmEdge pen (Vector Laboratories). It was important that tissue sections not dry out from this point. Sections were blocked with H₂O₂ for 10 minutes at room temperature. Slides were submerged in target retrieval solution at a temperature just below boiling for 10 minutes, rather than the manufacturer recommended boiling for 15 minutes. Protease plus solution was added to slides and incubated in HybEZ oven (ACD Bio) at 40°C for 10 minutes, rather than the manufacturer recommended 30 minutes. Target probe was added and allowed to hybridize for two hours at 40°C in the HybEZ oven. Serial steps to amplify signal involved the following solutions and conditions: Amp1 for 30 minutes at 40°C, Amp2 for 15 minutes at 40°C, Amp3 for 30 minutes at 40°C, Amp4 for 15 minutes at 40°C, Amp5 for 30 minutes at room temperature, Amp6 for 15 minutes at room temperature, and detection reagent for 10 minutes at room temperature. Slides were washed in wash buffer between each probe hybridization and amplification step. Slides were counterstained with 1:1 Gill's hematoxylin and water, allowed to air dry, and coverslipped with mounting media. Slides were imaged on an Aperio ImageScope CS2.

Expression Quantification and Statistics:

Representative images were selected from n=6 mammary carcinomas and n=6 non-diseased mammary tissues that were either immediately FFPE processed or whole-mounted and carmine stained prior to standard processing. Mean probe *per* epithelial/neoplastic cell was measured within outlined mammary ducts and cancerous lesions for *DapB*, *Mier3*, and *Polr2a* expression using the HALO-ISH quantification software (Indica Labs). Mean $\pm$ standard deviation of each RNAscope probe were used for quantitative comparisons. Two independent DMBA groups were sampled for mammary gland or mammary carcinoma tissue. Each tissue type was analyzed separately by Two-Way ANOVA. Independent variables were processing method (2 levels) and RNAscope probe (3 levels). Dependent variable was gene expression level measured as RNAscope target probe per cell. Tukey's *post hoc* test was used for group comparisons. Statistical analysis was performed using GraphPad Prism, version 7.00 for Windows (GraphPad Software).

Results & Discussion:

Alum carmine stained mammary gland whole-mount screening was used to detect various lesions known to develop during mammary cancer progression, including pre-malignant hyperplasia and pre-invasive ductal carcinoma *in situ* (DCIS) not detectable by palpation. Mammary ductal structures, interspersed within stromal tissue, were visible upon staining with alum carmine. Early hyperplastic proliferative lesions were detected, as expected, in alum carmine stained mammary gland whole-mounts (Fig 23A) [449, 450, 462]. The gross morphology of hyperplasia was extended, thickened, and amplified ductal branches (Fig 23B), that appeared as ductal proliferation of more than two cell layers (Fig

23C). Ductal carcinoma *in situ* (DCIS), an intraductal proliferation of epithelial cells, [453] was evident in whole-mounts as darkened structures (Fig 23A). These tumor lesions appeared as darkened structures due a dense concentration of neoplastic epithelial cells inhabiting these diseased morphologies (Fig 23D). Staining of tissue sections with H&E was used to confirm morphological classifications made in mammary gland whole-mounts. Tissue sections of lesions were also used to identify specific histopathological subtypes of excised lesions. Mammary tumor lesions appeared as a population of cells with characteristics of a papillary growth pattern (Fig 23E). Invasive ductal carcinoma (IDC) presented in tissue sections as migratory epithelial cells penetrating ductal walls and invading stroma (Fig 24) [391, 453]. These IDCs appeared as palpable mammary tumors that were visible without alum carmine staining.

Whole-mount processing and screening for lesions of interest, followed by RNAscope, was successfully used in this study to produce RNAscope results similar to results obtained using samples directly processed by FFPE, which is the recommended protocol. Exposure to additional reagents prior to FFPE processing, required for whole-mount preparation, did not have a negative effect on mRNA quality or abundance. No positive staining for the bacterial gene *four-hydroxy-tetrahydronicotinate reductase* (*DapB*) was observed (Fig 24). This indicated our negative control functioned properly as there was no signal for *DapB* RNA, which was not expected to be present. To test the sensitivity of RNAscope following whole-mount preparation and screening, we targeted the *Mier family member 3* (*Mier3*) gene, a relatively low to moderately expressed gene transcript in rat mammary and female breast tissue, with average expression of 11 transcripts per million [463]. For comparison, we probed for *RNA Polymerase II Subunit*

Figure 23: Premalignant and malignant lesions were visible in mammary gland tissue whole-mounts following alum carmine staining. A: A representative mammary gland from a Wistar Furth (WF/Hsd) virgin female eight weeks following 7,12-dimethylbenz(a)anthracene (DMBA) induction of mammary carcinogenesis. Hyperplasia appeared as dark thickened branching (black box). DCIS appeared as a dark mass within mammary fat pads (black arrow). **B:** Thickened hyperplastic ductal structures in a mammary fat pad. **C:** H&E staining of representative hyperplasia, indicated by proliferative ductal structures of more than two cell layers thick. **D:** A representative DCIS, indicated by a darkly stained tumor mass within the mammary fat pad. **E:** H&E staining of DCIS, indicated by a monoclonal expansion of cells in a papillary growth pattern.

Figure 24: Representative RNAscope results from *Mier3*, *Polr2a*, and *DapB* probes in tissue sections from rat mammary gland whole-mounts. Rat mammary gland ductal hyperplasia, ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) were identified in formalin-fixed alum-carmines stained whole-mounts. These lesions were excised and processed for RNAscope assays targeting *Mier family member 3* (*Mier3*), a moderately expressed gene (left column), *RNA Polymerase II Subunit A* (*Polr2a*), a highly expressed gene (middle column), and bacterial *4-hydroxy-tetrahydronicotinate reductase* (*DapB*), a negative control gene (right column). Each red puncta represents a single mRNA transcript [446]. Tissues were counterstained with hematoxylin. Images were taken at 40x magnification on an Aperio ImageScope CS2.

A (Polr2A), a relatively highly expressed gene with an average of 79 transcripts per million in human breast tissue [463]. RNAscope assays probing for *Polr2a* and *Mier3* reflected these expected expression differences (Fig 24). Results for *Polr2a* were densely packed red puncta within mammary ducts, while a probe targeting *Mier3* resulted in more scattered red puncta. There was no discernable difference in puncta abundance between RNAscope assays performed on samples excised from carmine stained whole-mounts (Fig 24) and mammary glands processed by FFPE immediately following tissue resection (Fig 25). This lack of difference between tissue treatment methods, along with an expected difference between independent gene target probes, indicated that the pre-screening process used did not negatively impact mRNA stability or abundance.

To determine if similar quantitative results of puncta density were obtained from different tissue processing methods, RNAscope images were quantified using HALO-ISH software (Indica Labs) and compared. Non-diseased mammary ducts had levels of *DapB* probe *per* mammary epithelial cell of 0.28 ± 0.26 by immediate FFPE and 0.35 ± 0.19 in tissue from whole-mounts (Fig 26). An RNAscope probe for *Mier3* gave results of 2.34 ± 0.46 in immediate FFPE tissue and 2.32 ± 0.14 in tissue processed from whole-mounts (Fig 26). The *Polr2a* probe per mammary epithelial cell were 7.55 ± 1.22 in immediately processed FFPE tissue and 7.21 ± 1.22 in tissue from whole-mounts (Fig 26). There was no statistically significant difference in quantities between tissues that were immediately FFPE processed and tissues excised from mammary gland whole-mounts. There was, as expected, a statistically significant difference between gene probes ($p < 0.0001$). This confirmed anticipated results of no *DapB* expression, moderate *Mier3* expression in both

Figure 25: Representative images of RNAscope assays using *Mier3*, *Polr2a*, and *DapB* probes in rat mammary gland and mammary carcinoma tissue. Immediately processed FFPE tissue samples from non-diseased mammary glands or DMBA-induced mammary carcinomas were used. RNAscope assay probes for *Mier family member 3* (*Mier3*), a moderately expressed gene (left column), *RNA Polymerase II Subunit A* (*Polr2a*), a highly expressed gene (middle column), and bacterial *4-hydroxy-tetrahydrodipicolinate reductase* (*DapB*), a negative control gene (right column), were targeted. Each red puncta represents a single mRNA transcript [446]. Tissues were counterstained with hematoxylin. Images were taken at 40x magnification on an Aperio ImageScope CS.

A Non-diseased Mammary Ducts

B Mammary Carcinomas

Figure 26: Quantitative analysis of RNAscope assays. RNAscope assays were performed on non-diseased mammary gland (n=6; panel A) and mammary carcinoma (n=6, panel B) tissue from the same rats. Tissues were either immediately processed by FFPE (manufacturer recommended) or excised from whole-mounts prior RNAscope assays. Tissue sections were probed for *Mier family member 3 (Mier3)*, a moderately expressed gene, *RNA Polymerase II Subunit A (Polr2a)*, a highly expressed gene, and bacterial *4-hydroxy-tetrahydrodipicolinate reductase (DapB)*, a negative control gene that is not present in mammalian tissues. RNAscope probes per cell in non-diseased mammary ducts and mammary carcinomas were quantified using HALO-ISH quantification software (Indica Labs). There was no significant effect of processing method within either tissue type (Panel A- p=0.5057, Panel B- p=0.6086). There was a significant effect of RNAscope probe in both tissue types (Panels A and B p values <0.0001). RNAscope probe gene targets with different lowercase letters were significantly different (p<0.05).

tissue processing methods, and high *Polr2a* expression, which indicated the quantitative sensitivity of RNAscope assays were retained in samples taken from whole-mounts.

Similar quantitative results between tissue processing methods were obtained when RNAscope assays from mammary carcinomas were analyzed (Fig 26). The *DapB* probe *per* carcinoma cell results were 0.15 ± 0.17 in immediate FFPE tumors and 0.23 ± 0.20 in mammary tumors taken from whole-mounts. The *Mier3* probe per cell results were 4.45 ± 0.64 in immediate FFPE tumor tissue and 4.30 ± 0.59 in tumor tissue from whole-mounts. The *Polr2a* probe resulted per cell quantities of 12.27 ± 1.65 in immediate FFPE processed carcinomas and 11.52 ± 1.90 in carcinoma tissue from whole-mounts. There was no statistically significant effect of tissue processing method; however, there was a significant effect of RNAscope target probe ($p < 0.0001$). These results validated that RNAscope was a robust and appropriate procedure for quantitative analysis of gene expression in mammary carcinoma tissue excised from mammary gland whole-mounts.

Conclusion:

RNAscope was used successfully on tissue collected from mammary gland whole-mounts that had been processed for carmine staining. There was no loss in qualitative or quantitative gene expression measurements using RNAscope on tissue excised from whole-mounts. This method allowed for *in situ* pre-screening of mammary tissue to identify lesions or morphological epithelial changes that were visually detectable after whole tissue carmine staining. Methods outlined in this report provide a procedure to identify and quantitatively evaluate gene expression in various mammary gland pathologies associated with disease progression.

CHAPTER V

MIER3 EXPRESSION INCREASES THROUGHOUT RAT MAMMARY CANCER PROGRESSION

Introduction:

The human breast contains small alveolar buds at the distal ends of ducts that are collectively referred to as terminal ductal lobular units. In the rat mammary gland these distal ductal buds are referred to as terminal end buds. It is commonly hypothesized that these structures are the initiation sites of invasive ductal carcinomas as they are the most rapidly proliferative components of the mammary gland, and thus contain vulnerable precursor cells [102, 464]. Ductal carcinomas, which comprise the majority of breast cancers, are believed to develop along a precursor-carcinoma sequence [218, 465, 466] beginning with normal ducts which can initiate proliferative disease. Disease progression of ductal origin can develop in sequential stages characterized by increasing cellular abnormalities and proliferation [465, 466]. This simplified model is also applicable to DMBA-induced rat mammary cancer. In fact, the earliest observable change in rat mammary tissue following DMBA administration is atypical hyperplasia of cells surrounding ducts and ductules, which is the earliest disease morphology of the progression model [219].

Normally ducts are lined by two cell layers, the inner luminal cell layer and the

outer myoepithelial layer. Proliferative cells can increase the cell number of this bi-cell layer, referred to as hyperplasia [467]. Usual ductal hyperplasia comprises a still heterogeneous population of cells that retains the ductal structure, and while it carries a 1.5 relative risk for development of invasive breast cancer, it does not significantly increase the risk of breast cancer development [467-469]. Atypical ductal hyperplasia (ADH) is a premalignant stage of the precursor-carcinoma disease sequence characterized by excessive proliferation of the mammary epithelium. In this stage, an outgrowth of the luminal epithelial cell layer is morphologically distinct from usual hyperplasia through clonal expansion of a now uniform population of cells, which can sometimes encompass the entire luminal space and distort the ductal architecture [465, 467, 470]. ADH can progress to ductal carcinoma *in situ* (DCIS), which is a preinvasive stage characterized by what is now considered neoplastic proliferation of epithelial cells that are still retained within the ductal-lobular structures [465]. ADH and low-grade DCIS share the same low grade monoclonal cell population, but ADH partially fills duct spaces whereas DCIS completely fills the duct spaces. Distinguishing between ADH and low-grade DCIS is based on quantitative criteria, with classification of a lesion as low-grade DCIS requiring complete involvement of at least two duct spaces or completely filled duct spaces measuring >2 mm [465, 470, 471]. Histologically, DCIS is considered to progress to invasive ductal carcinoma (IDC) when this lesion has extended through the basement membrane and invaded into the surrounding stroma [465]. This stage is characterized by a fragmented myoepithelial layer and basement membrane, if present at all [472]. IDC is a dangerous form of cancer as it can lead to metastasis and the formation of secondary tumors [472, 473]. In summary, this precursor-carcinoma sequence of breast cancer development

Figure 27: Precursor-Carcinoma Sequence of Mammary Cancer Development: A normal mammary duct is bilayered, with an inner luminal cell layer (light pink) and an outer myoepithelial cell layer (dark pink), and a basement membrane (gray circle) that separates the epithelial components from the outside stroma and adipose tissue. Non-cancerous usual ductal hyperplasia occurs when luminal cells proliferate and increase the cell numbers from a bilayer. Atypical ductal hyperplasia (ADH) is a premalignant stage that is a result of clonal expansion of the luminal epithelial cell layer (bright pink) and outgrowth into the luminal space. ADH can progress to the preinvasive stage ductal carcinoma *in situ* (DCIS). This is a further expanded lesion from neoplastic cells but is still retained by the myoepithelial layer and basement membrane. DCIS is classified as an invasive ductal carcinoma (IDC) when the myoepithelial cell layer begins to disintegrate and the malignancy has extended through the basement membrane and invaded into the surrounding stroma.

is comprised of definitive pathological stages, initiating as a premalignant stage of ADH, progressing to a preinvasive stage of DCIS, and culminating in the potentially lethal stage of IDC (Fig 27) [218, 464, 466].

Cell proliferation drives a cancer phenotype. The average proliferation rate of terminal ductal lobular units, the most proliferative structure in the non-diseased breast is 2%, which rises to 5% in ADH and 15% in DCIS [471]. It is widely held that cells acquire proliferative and malignant phenotypes through the accumulation of genetic activations/inactivations and mutations throughout cancer progression [474]. Transcriptional analysis of normal breast epithelium as compared to ADH, DCIS, and IDC has revealed distinct global alterations in gene expression shared amongst premalignant and malignant disease [464, 466, 470, 475]. The finding that gene expression profiles are highly similar at each of these stages of breast cancer progression suggests a common clonal origin and that genes conferring proliferative and invasive potential are active at premalignant and preinvasive stages and maintained through later disease stages [464, 470, 476]. As a result, the transcriptome of an early disease stage could be a useful indicator of the invasive potential of a later pathological lesion [464].

Previous work identified *Mier3* as a candidate mammary cancer susceptibility gene through physical mapping of the rat mammary cancer susceptibility quantitative trait locus, *Mcs1b*. This locus was narrowed to a 1.8Mb region on rat chromosome 2 and results in around a 50% reduction in DMBA-induced mammary tumor multiplicity in rats homozygous for the Copenhagen strain *Mcs1b* resistance allele [248]. There are two genes encompassed in the overlap between rat *Mcs1b* and established human breast cancer risk allele 5q11.2, *MIER3* and *Mitogen-Activated Protein Kinase Kinase 1* (*MAP3K1*).

MAP3K1 is an established breast cancer gene [477]. In this study, I used a DMBA-induced rat mammary cancer model of female breast cancer to investigate *Mier3* expression throughout a precursor-carcinoma sequence of ductal origin. I report the predominant *Mier3*-expressing mammary gland cell type as ductal luminal epithelial cells, significant increases in *Mier3* expression with increasing malignancy of each histopathological stage of progression, and the highest *Mier3* levels in invasive ductal carcinomas. These findings suggest a potential role in disease progression and elevate MIER3 as a possible biomarker or therapeutic target in breast disease development.

Methods:

Animal husbandry

Rats were maintained in an AAALAC-approved facility on a 12 h light/dark cycle and provided LabDiet 5001 Rodent Diet (PMI® Nutrition International) and water ad libitum. Inbred Wistar Furth (WF/Hsd) females were obtained from Envigo. All animal protocols were approved by the University of Louisville Animal Care and Use Committee.

Congenic Strains and Animal Breeding

The Wistar Furth (WF/NHsd) inbred rat strain was used as a mammary cancer susceptible line. The Wistar Furth.Copenhagen (WF.COP) congenic rat strain N3 contains a segment of Copenhagen (COP/NHsd) on *RNO2* from markers D2Mgh2 to g2UL1-5 (Chr2:40171863 – 43756834, build RGSC 6.0/rn6) and is positive for the *Mcs1b* decreased susceptibility allele. The *Mcs1b* N3 strain at generation N16F40 was used as a mammary cancer resistance line and compared to the susceptible WF strain.

DMBA Administration and Mammary Gland Tissue Collection

At 50-55 days of age 7,12-Dimethylbenz(a)anthracene (DMBA Acros Organics, CAS#: 57-97-6, 20 mg/mL in sesame oil) was given by single oral gavage (65 mg DMBA/kg body mass) to WF and WF.COP-*Mcs1b* strain N3 females to induce mammary carcinogenesis. At time points of 4, 8, 12, and 15 weeks following DMBA administration, six rats of each strain were euthanized and mammary glands formalin fixed and paraffin embedded (FFPE) (Fig 28).

Tissue selection and Classification

Sectioning of FFPE blocks was done one day *prior* to a planned RNAscope assay, as mRNA remains more stable within paraffin blocks than tissue sections [460]. Mammary gland FFPE blocks were cut in 4 um sections onto Superfrost Plus microscope slides using a rotary microtome (Thermo HM 355S). Representative slides from mammary glands exposed to DMBA were stained by hematoxylin and eosin to identify histopathological features of mammary cancer progression. Minimally 6 slides from each *Mcs1b* genotype that displayed normal mammary duct morphology and usual hyperplasia were selected to assay within the timepoint of 4 weeks post-DMBA administration. At least 6 slides per genotype within each of the remaining carcinogenesis timepoints of 8, 12, and 15 weeks post-DMBA administration were selected to assay that displayed stages of progression – normal morphology, usual hyperplasia, ADH, DCIS, and IDC.

Hematoxylin and Eosin (H&E) staining of sectioned mammary glands:

An H&E stain on sectioned tissues was used to assess tissue sample quality and to confirm the lesion of interest was present in the section. Slides were deparaffinized and rehydrated using 3 separate xylene washes followed by single washes each in 100% ethanol, 90% ethanol, 80% ethanol, and DI water. Tissues were stained with hematoxylin for 3 minutes, rinsed under running DI water for 3 minutes, soaked in bluing reagent for less than a minute, and again rinsed with DI water. Slides were dipped a few times in 100% ethanol to eliminate water before staining in eosin-Y for 30-45 seconds. Tissue was then dehydrated using washes of 90% ethanol, 100% ethanol, and 3 separate xylene washes. Samples were air dried and coverslipped using permount or similar mounting media.

RNA in situ hybridization

RNAscope was used to identify the spatiotemporal cell-specific expression of *Mier3* in rat mammary tissue according to manufacturer's directions (ACD Bio). Briefly, slides were deparaffinized in xylene and 100% ethanol, pretreated with an H₂O₂ block for 10 minutes, placed in target retrieval solution that was just under boiling (ACD Bio 322000) for 10 minutes, and treated with protease plus solution for 10 minutes (ACD Bio 322330). The target probe was added and slides were incubated in HybEZ oven (ACD Bio) at 40°C for 2 hours. Amplification steps were as follows using an RNAscope 2.5 HD red detection kit (ACD Bio 322360): Amp1 for 30 minutes at 40°C, Amp2 for 15 minutes at 40°C, Amp3 for 30 minutes at 40°C, Amp4 for 15 minutes at 40°C, Amp5 for 30 minutes at room temp, Amp6 for 15 minutes at room temp, and detection reagent for 10 minutes at room temp. Slides were counterstained with 1:1 Gill's hematoxylin and water. Vectashield

mounting media (Vector Laboratories H-1000) was added to protect staining. Slides were imaged at 40x on an Aperio ImageScope CS2.

Expression Quantification, Data Handling, and Statistical Analysis

Within images of tissue slides following the RNAscope assay, occurrences of normal mammary ducts, usual hyperplasia, ADH, DCIS, and IDC were outlined and selected for analysis using the HALO-ISH quantification software from Indica Labs. Mean probe per mammary epithelial cell and percent of Mier3 positive mammary epithelial cell data was collected. Average *Mier3* transcript quantification values were transformed by taking the natural log. Percent positivity values were transformed by taking the arcsin of the square root of the decimal percent values. Transformed data were analyzed via one-way or two-way ANOVA depending on the number of variables. Independent variables included timepoint, genotype, and morphology. All statistical analysis was done using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

Results:

The RNAscope assay functions properly in rat mammary gland and disease tissue

To study *Mier3* expression throughout mammary cancer progression, WF susceptible and WF.COP *Mcs1b*-resistant N3 females were administered DMBA and mammary glands analyzed at various timepoints following this exposure. A mammary gland collection timeline of all experimental stages used in this study is outlined in Figure 28. To begin, the utility of the RNAscope assay in rat mammary tissue was tested using the experimental Mier3 probe and manufacturer recommended (ACD Bio) control probes. A

Figure 28: Disease progression mammary gland collection timeline: Female rats from the WF and WF.COP *Mcs1b* N3 strain were administered the chemical carcinogen DMBA at 50-55 days of age. Mammary glands were collected at 4, 8, 12, and 15 weeks post administration (top row), corresponding to 12, 16, 20, and 23 weeks of age (bottom row).

probe for *RNA Polymerase II Subunit A (Polr2a)* was used as a positive control due to its ubiquitous expression and the probe for the bacterial gene *4-hydroxy-tetrahydrodipicolinate reductase (DapB)* used as a negative control. Mammary tissue has a high adipose content so is not as robust as other tissue types. As a result, the manufacturers protocol had to be experimentally modified to reduce the harsh pre-treatment conditions, namely lower temperature of the target retrieval buffer, and less exposure time to the buffer and protease treatment. Following optimization of the protocol, all three probes functioned as expected in each progression stage using the RNAscope assay, with indistinct stains due to high *Polr2a* expression, median *Mier3* expression appearing as distinct puncta, and no *DapB* expression. This demonstrates the specificity of the *Mier3* probe in rat mammary tissue (Fig 29).

Mammary epithelial cells are the highest *Mier3*-expressing cells in the rat mammary gland

To determine if there is cell-specific expression of *Mier3* in rat mammary glands, RNAscope assays probing for *Mier3* followed by quantification with HALO-ISH module software from Indica Labs were performed on mammary glands from 8-week-old WF strain females. Regions of assayed mammary glands were classified according to major tissue elements (Fig 30A) and collectively quantified (Fig 30B) (Table 18). Mammary ducts displayed the greatest average *Mier3* probe per mammary epithelial cell (MEC) followed by lymph nodes. Blood vessels, stroma, and adipose tissue showed negligible *Mier3* expression. One-way ANOVA followed by Tukey's multiple comparisons test was performed. No difference was found between mammary ducts and lymph nodes, as well as

Figure 29: RNA *in situ* hybridization (RNAscope) control and Mier3 probes function properly in rat mammary tissue: Shown are representative images of the ductal histopathologies along the precursor-carcinoma sequence of mammary cancer progression following RNAscope assays (top to bottom). Columns display assays that probed for Mier3, 4-hydroxy-tetrahydrodipicolinate reductase probe (DapB, negative control), and RNA Polymerase II Subunit A probe (Polr2A, positive control). Images were taken at 40x magnification on an Aperio ImageScope CS2.

A

**Mammary
Duct**

**Lymph
Node**

**Blood
Vessel**

Stroma

Adipose

B

Mier3 expression in rat mammary gland tissue elements

Figure 30: Mammary ducts possess the greatest *Mier3* expression levels of all rat mammary gland tissue elements: RNAscope assays probing for *Mier3* were done on 8-week-old WF female mammary glands.

A- 40x mammary gland histologic images show red puncta (*Mier3* transcripts) counterstained with hematoxylin. Rows display a representative mammary duct, lymph node, blood vessel, stroma, and adipose tissue. The right column is the red probe and nuclei markup from the HALO-ISH module software from Indica Labs. Negative cells are white. Cells positive for *Mier3* are shades of red, with light pink corresponding to 1-3 puncta and dark red displaying >14 puncta.

B- Quantification of mean *Mier3* expression per cell analyzed in each mammary tissue element. One-way ANOVA followed by Tukey's multiple comparisons test. Connected morphology groups indicate Tukey's *post hoc* test $p < 0.0001$. $n=6$ individual rat mammary glands, $n=2$ viewpoints per sample, for $N=12$ per tissue element.

Table 18. Spatial expression of *Mier3* in 8-week-old mammary gland tissue from female WF/NHsd rats

Tissue Element	Cell Types	Mean probe per cell (SD)	Percent of positive cells (SD)
Mammary Ducts	Myoepithelial, luminal epithelial cells	2.66 (0.24)	58.2 (8.1)
Lymph Nodes	Immune cells	1.44 (0.43)	32.4 (8.5)
Blood Vessels	Vascular endothelial cells	0.27 (0.16)	10.2 (5.3)
Stroma	Fibroblasts	0.12 (0.06)	5.3 (2.8)
Adipose	Adipocytes	0.07 (0.04)	4.1 (2.0)

n= 6 mammary glands, n=2 viewpoints per tissue element, N=12

between blood vessels, stroma, or adipose. However, there was a significant difference between these two groups ($p < 0.0001$). In the mammary ducts specifically, the inner luminal epithelial cell layer visibly possesses the majority of *Mier3* expression, with the outer myoepithelial cell layer displaying nominal expression (Fig 31).

DMBA induces *Mier3* expression in non-diseased rat mammary glands

Following the finding that MECs in mammary ducts are the major *Mier3*-expressing cell type in rat mammary glands, the effect of administration of the mammary carcinogen DMBA on *Mier3* expression was explored. Mammary glands were collected from 12-week-old age-matched WF strain females either unexposed (control) or 4 weeks post-DMBA administration. This age was selected for tissue collection because it follows the acute phase for DMBA-toxicity but is also prior to the development of mammary lesions and carcinomas in susceptible rat strains. RNAscope assays probing for *Mier3* and downstream HALO-ISH module software quantification was performed on mammary ducts (Fig 32A) (Table 19). DMBA-exposed mammary ducts had significantly greater *Mier3* expression levels (unpaired 2-tailed t test $p = 0.0001$) (Fig 32B). DMBA-exposed MECs also had greater percent positivity for overall *Mier3* expression (unpaired 2-tailed t test $p = 0.0001$) (Fig 32C).

There is no difference in *Mier3* expression between bi-layered and hyperplastic mammary ducts

After determining an effect of DMBA administration of *Mier3* expression, *Mier3* expression differences were explored throughout mammary cancer progression in

Figure 31: The majority of ductal *Mier3* expression is localized to luminal epithelial cells. This representative mammary duct histologic image shows red puncta (*Mier3* transcripts) counterstained with hematoxylin. Image was taken at 40x magnification on an Aperio ImageScope CS2. Black arrows point to outer myoepithelial cells that show nominal *Mier3* expression. Inner columnar luminal epithelial cells display the greatest *Mier3* expression levels.

A

12 weeks Control

12 weeks +DMBA

B

Ductal *Mier3* expression in rat mammary glands at 12 weeks of age with and without DMBA exposure

C

***Mier3* positive MECs in rat mammary glands at 12 weeks of age with and without DMBA exposure**

Figure 32: Ductal *Mier3* expression is significantly increased with DMBA-exposure:

RNAscope assays probing for *Mier3* were done on age-matched 12-week-old WF female rat mammary glands that were either unexposed to DMBA (control) or administered DMBA 4 weeks prior. **(A)** 40x mammary gland histologic images show red puncta (*Mier3* transcripts) counterstained with hematoxylin. Columns display an unexposed mammary duct (left) and a DMBA-exposed mammary duct (right). The bottom row is the red probe and nuclei markup from the HALO-ISH module software. **(B)** Quantification of mean *Mier3* expression per MEC. There was a significant difference in expression with DMBA exposure using a one-way ANOVA followed by Tukey's *post hoc* test ($p=0.0001$). **(C)** Quantification of MECs positive for *Mier3* expression. There was a significant difference in *Mier3* positive cells with DMBA exposure using an unpaired 2-tailed t test ($p=0.0001$). $N=12$ mammary glands per group.

Table 19. *Mier3* expression in 12-week-old female WF/NHsd rat mammary glands with and without DMBA exposure

Treatment	Mean probe per cell (SD)	Percent of positive cells (SD)
None	1.03 (0.28)	41.3 (8.2)
DMBA	2.24 (0.68)	59.4 (8.5)

N=12 mammary glands per group

mammary cancer resistant vs susceptible strains of rat. Mammary glands from the WF and WF.COP *Mcs1b*-N3 strains of rats were first collected at an early timepoint of 4 weeks following administration of DMBA. RNAscope assays probing for *Mier3* transcripts was performed and resulting images quantified using the HALO-ISH module software from Indica Labs (Fig 33A) (Table 20). Mammary ducts were classified as normal based on a typical bi-cell layer or usual ductal hyperplasia if there was an increase in cell number across the duct. There was no significant difference in *Mier3* expression based on genotype or morphology ($p=0.3604$ genotype, $p=0.3661$ morphology, two-way ANOVA) (Fig 33B). There was also no difference when quantifying based on MEC *Mier3* percent positivity ($p=0.4048$ genotype, $p=0.1793$ morphology, two-way ANOVA) (Fig 33C).

***Mier3* expression significantly increases with each histopathological stage along a precursor-carcinoma sequence of mammary cancer development**

To continue *Mier3* expression analysis throughout disease progression, the process of mammary gland tissue collection, RNAscope assays, and quantification using HALO-ISH module software was performed at 8-, 12-, and 15-weeks post-DMBA administration in WF and WF.COP *Mcs1b*-N3 females. At these timepoints, IDC and the morphologies of the precursor-carcinoma sequence that can lead to IDC are present, including ADH and DCIS. These progression morphologies, as well as normal mammary ducts and hyperplastic ducts, were assessed for *Mier3* expression (Fig 34A, 35A, 36A).

A

B

***Mier3* expression in rat mammary glands
4 weeks post DMBA administration**

Figure 33: *Mier3* expression in rat mammary glands 4 weeks post DMBA administration: (A) 40x mammary gland histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. Columns display a representative non-diseased mammary duct (left) and a mammary duct with usual hyperplasia (right). The middle row provides a closer view to observe red puncta. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in normal mammary ducts and ducts with usual hyperplasia in the WF vs N3 strain of rat. Two-way ANOVA followed by Tukey's multiple comparisons test found no significant difference based on genotype or morphology. (C) Quantification of MECs positive for *Mier3* expression. Two-way ANOVA followed by Tukey's multiple comparisons test found no significant difference based on genotype or morphology. N=6 mammary glands per genotype, minimally n=8 viewpoints per morphology per genotype.

Table 20. *Mier3* mammary ductal expression in WF/NHsd and WF.COP *Mcs1b*-N3 females 4 weeks post-DMBA administration

Genotype	Morphology	Mean probe per cell (SD)	Percent of positive cells (SD)
WF/NHsd	Bi-layered ducts	2.27 (0.73)	59.6 (9.1)
WF.COP <i>Mcs1b</i> -N3	Bi-layered ducts	2.15 (0.53)	59.0 (6.7)
WF/NHsd	Hyperplastic ducts	2.18 (0.67)	60.3 (8.5)
WF.COP <i>Mcs1b</i> -N3	Hyperplastic ducts	2.56 (0.38)	65.2 (6.7)
N=6 mammary glands per genotype			

Quantification of mean Mier3 probe per MEC/carcinoma cell at 8 weeks post-DMBA administration found no significant difference based on genotype, but there was for morphology ($p=0.4096$ genotype, $p<0.0001$ morphology, two-way ANOVA) (Fig 34B) (Table 21). Normal/hyperplastic ducts were significantly different from ADH ($p=0.0001$, Tukeys *post hoc*) and DCIS and IDC ($p<0.0001$). ADH significantly differed from IDC ($p=0.01$). Overall cells positive for *Mier3* was also assessed ($p=0.0552$ genotype, $p<0.0001$ morphology, two-way ANOVA) (Fig 34C). Normal/hyperplastic ducts differed from ADH ($p=0.0001$), DCIS ($P<0.0001$), and IDC ($p=0.001$). ADH differed from IDC ($p=0.01$).

At 12 weeks post-DMBA administration, there was again only a difference based on mammary gland disease morphology when analyzing by mean Mier3 probe per cell ($p=0.6544$ genotype, $p<0.0001$ morphology, two-way ANOVA) and overall positivity ($p=0.2624$ genotype, $p<0.0001$ morphology, two-way ANOVA) (Fig 35B&C) (Table 22). With both quantification methods, there was a significant difference between normal/hyperplastic ducts and ADH, DCIS, and IDC ($p<0.0001$, Tukeys *post hoc*).

In the final mammary cancer progression timepoint of 15 weeks post-DMBA administration, quantification once again only yielded a difference based on disease morphology by mean probe per cell analysis ($p=0.5837$ genotype, $p<0.0001$ morphology, two-way ANOVA) and overall Mier3 positive cells ($p=0.5470$ genotype, $p<0.0001$ morphology, two-way ANOVA) (Fig 36B&C) (Table 23). With both quantification methods, there was a significant difference between normal/hyperplastic ducts and ADH, DCIS, and IDC ($p<0.0001$, Tukeys *post hoc*). When analyzing mean probe per cell there was also a difference between ADH and IDC ($p=0.001$), but this was lost when assessing overall positivity.

A

B *Mier3* expression in rat mammary glands
8 weeks post DMBA administration

C *Mier3* positive cells in rat mammary glands
8 weeks post DMBA administration

Figure 34: *Mier3* expression in rat mammary glands 8 weeks post DMBA administration. (A) 40x mammary gland histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. Columns display (from left to right) representative non-diseased duct, hyperplastic duct, ADH, DCIS, and IDC. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC or cancer cell in the WF vs N3 strain. (C) Quantification of *Mier3*-positive cells in a set field of view of MECs/cancer cells. In both graphs morphology groups with different lowercase letters are significantly different at Tukey's *post hoc* test $p < 0.01$. N=6 mammary glands per genotype, minimally n=6 viewpoints per morphology per genotype

Table 21. *Mier3* expression by mammary cancer progression morphologies in WF/NHsd and WF.COP *Mcs1b*-N3 females 8 weeks post-DMBA administration

Genotype	Morphology	Mean probe per cell (SD)	% of positive cells (SD)
WF/NHsd	Non-diseased ducts	2.48 (0.56)	59.2 (7.0)
WF.COP <i>Mcs1b</i> -N3	Non-diseased ducts	2.69 (0.23)	62.1 (4.4)
WF/NHsd	Hyperplastic ducts	2.48 (0.92)	55.6 (11.7)
WF.COP <i>Mcs1b</i> -N3	Hyperplastic ducts	2.60 (0.42)	57.8 (7.0)
WF/NHsd	ADH	3.74 (0.97)	73.3 (5.4)
WF.COP <i>Mcs1b</i> -N3	ADH	3.77 (0.88)	68.6 (5.9)
WF/NHsd	DCIS	4.34 (0.44)	79.8 (7.5)
WF.COP <i>Mcs1b</i> -N3	DCIS	4.30 (0.61)	68.7 (5.8)
WF/NHsd	IDC	5.04 (0.39)	70.0 (3.5)
WF.COP <i>Mcs1b</i> -N3	IDC	4.89 (0.46)	69.8 (4.1)

N=6 mammary glands per genotype

B *Mier3* expression in rat mammary glands
12 weeks post DMBA administration

C *Mier3* positive cells in rat mammary glands
12 weeks post DMBA administration

Figure 35: *Mier3* expression in rat mammary glands 12 weeks post DMBA administration. (A) 40x mammary gland histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. Columns display (from left to right) representative non-diseased duct, hyperplastic duct, ADH, DCIS, and IDC. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC or cancer cell in the WF vs N3 strain. (C) Quantification of *Mier3*-positive cells in a set field of view of MECs/cancer cells. For both graphs morphology groups with different lowercase letters indicate Tukey's *post hoc* test $p < 0.0001$. N=6 mammary glands per genotype, minimally n=6 viewpoints per morphology per genotype.

Table 22. *Mier3* expression by mammary cancer progression morphologies in WF/NHsd and WF.COP *Mcs1b*-N3 females 12 weeks post-DMBA administration

Genotype	Morphology	Mean probe per cell (SD)	% of positive cells (SD)
WF/NHsd	Non-diseased ducts	2.22 (0.44)	48.5 (7.7)
WF.COP <i>Mcs1b</i> -N3	Non-diseased ducts	2.26 (0.53)	50.3 (8.6)
WF/NHsd	Hyperplastic ducts	2.23 (0.65)	49.3 (7.6)
WF.COP <i>Mcs1b</i> -N3	Hyperplastic ducts	2.20 (0.65)	48.6 (11.3)
WF/NHsd	ADH	3.64 (0.68)	68.1 (9.5)
WF.COP <i>Mcs1b</i> -N3	ADH	3.56 (0.74)	71.2 (10.0)
WF/NHsd	DCIS	3.98 (0.68)	65.2 (6.8)
WF.COP <i>Mcs1b</i> -N3	DCIS	4.04 (0.49)	70.3 (5.5)
WF/NHsd	IDC	4.76 (1.12)	70.2 (10.4)
WF.COP <i>Mcs1b</i> -N3	IDC	4.31 (0.65)	69.7 (5.9)

N=6 mammary glands per genotype

A

B *Mier3* expression in rat mammary glands
15 weeks post DMBA administration

C *Mier3* positive cells in rat mammary glands
15 weeks post DMBA administration

Figure 36: *Mier3* expression in rat mammary glands 15 weeks post DMBA administration. (A) 40x mammary gland histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. Columns display (from left to right) representative non-diseased duct, hyperplastic duct, ADH, DCIS, and IDC. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC or cancer cell in the WF vs N3 strain. (C) Quantification of *Mier3*-positive cells in a set field of view of MECs/cancer cells. For both graphs morphology groups with different lowercase letters indicate Tukey's *post hoc* test $p < 0.0001$. N=6 mammary glands per genotype, minimally 8 viewpoints per morphology per genotype.

Table 23. *Mier3* expression by mammary cancer progression morphologies in WF/NHsd and WF.COP *Mcs1b*-N3 females 15 weeks post-DMBA administration

Genotype	Morphology	Mean probe per cell (SD)	% of positive cells (SD)
WF/NHsd	Non-diseased ducts	2.19 (0.30)	50.2 (6.1)
WF.COP <i>Mcs1b</i> -N3	Non-diseased ducts	2.27 (0.34)	47.0 (4.8)
WF/NHsd	Hyperplastic ducts	2.21 (0.31)	49.6 (6.5)
WF.COP <i>Mcs1b</i> -N3	Hyperplastic ducts	2.21 (0.43)	48.1 (4.8)
WF/NHsd	ADH	3.78 (0.60)	64.0 (3.61)
WF.COP <i>Mcs1b</i> -N3	ADH	3.88 (0.81)	71.9 (7.2)
WF/NHsd	DCIS	4.25 (0.71)	67.4 (7.0)
WF.COP <i>Mcs1b</i> -N3	DCIS	4.60 (0.82)	69.3 (8.5)
WF/NHsd	IDC	5.13 (0.70)	71.0 (5.1)
WF.COP <i>Mcs1b</i> -N3	IDC	4.97 (1.07)	68.3 (11.1)

N=6 mammary glands per genotype

Carcinoma cells display heterogenous *Mier3* expression in mammary tumors

In analyzing each progression stage based on histology as opposed to absolute quantification, there was an interesting appearance of IDCs, in which certain carcinoma cells express markedly increased levels of *Mier3* as compared to neighboring cells (Fig 37A). This was consistent across tumors taken at 8-, 12-, and 15-weeks post-DMBA administration. This heterogeneity in expression is observed throughout tumor sections (Fig 37B) and is seen in neoplastic cells but is not observed in healthy morphologies.

Summary: *Mier3* expression increases with greater malignancy of mammary lesions, but the overall number of cells expressing *Mier3* does not change

In the summation of the expression data, there were no statistically significant differences between pathology stages based on *Mcs1b* genotype and timepoint collected following DMBA administration. Data was pooled to provide a final quantification of mean *Mier3* expression (Fig 38A) and percent of *Mier3* positive cells (Fig 38B) within each histopathological stage of the precursor-carcinoma sequence of rat mammary cancer development. There was a significant increase in mean *Mier3* expression between non-diseased mammary ducts and cancerous lesions, as well as between each pathology stage ($p < 0.001$). There was also a significant increase in cells positive for *Mier3* expression between non-diseased mammary ducts and cancerous lesions ($p < 0.001$). However, there were no differences between pathology stages. In summary, there were no differences in expression based on time post-DMBA administration or *Mcs1b* genotype. However, there was an increase in *Mier3* expression with each increase in malignancy of histopathological

Figure 37: Rat mammary carcinoma cells express variable levels of *Mier3*:

Mammary carcinoma histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. Images were taken at 40x magnification on an Aperio ImageScope CS2. **(A)** Within various mammary IDCs, certain carcinoma cells express very high levels of *Mier3* (black arrows) as compared to neighboring cells. **(B)** This single IDC field of view provides an uncropped example of the intra-tumoral heterogeneity of *Mier3* expression.

Figure 38: *Mier3* expression in the histopathological stages of the precursor-carcinoma sequence of rat mammary cancer progression: Quantification by stage of disease progression for **(A)** mean *Mier3* probe per MEC/cancer cell and **(B)** Quantification of *Mier3*-positive cells in a set field of view of MECs/cancer cells. Data was pooled from 4, 8, 12, and 15 weeks post DMBA-administration and combined genotypes as there was no difference between morphologies based on time or genotype. Following a significant one-way ANOVA ($p < 0.0001$) different lowercase letters indicate significant Tukey's multiple comparisons tests ($p < 0.001$). Minimally $N=64$ quantified fields of view per morphology.

progression stage, with no differences in total *Mier3* positive cells between cancerous lesions.

Discussion:

When first analyzing the rat mammary gland for *in situ*, cell-specific expression of *Mier3*, I found nominal, if any expression, in vascular endothelial cells, fibroblasts, and adipocytes, indicating *Mier3* does not have a role in the stromal compartment of the mammary gland. I found the highest levels in mammary epithelial cells (MECs) that make up the mammary ducts. This is relevant to mammary cancer as the epithelial compartment is the most active component of the mammary gland and ductal structures are most susceptible to chemical insult [114, 117]. Terminal ductal lobular units are also the site of origin of invasive ductal carcinomas (IDC), which account for 80% of diagnosed breast cancers worldwide [218, 478]. When evaluating MECs specifically, luminal epithelial cells were found to express greater levels of *Mier3* as compared to myoepithelial cells. This is important to breast cancer etiology as the majority of breast cancers have luminal epithelial cell characteristics, making these cells the likely precursors for neoplastic transformation [117, 479]. This analysis provided crucial cell-specific expression information adding to the potential role of *Mier3* in mammary cancer by identifying its most prominent expression levels as stemming from the cell population that originates the majority of breast carcinomas.

I next examined the effect of carcinogen exposure on *Mier3* expression in rat mammary glands. There was two-fold greater *Mier3* expression in non-diseased mammary ducts of rats exposed to DMBA as compared to age-matched, non-exposed control

mammary glands. Changes in gene expression in DMBA-induced lesions has been analyzed for decades [480, 481], and minimally nominates these identified genes as markers of mammary carcinogenesis. However, our analysis was conducted at an early induction time point prior to the formation of pathological disease lesions, indicating a potential role of *Mier3* in disease initiation. Luminal epithelial cells are described as being on a path of continuous differentiation, with the population displaying variable levels of progenitor and differentiation markers [482], making them as a whole susceptible to carcinogen initiation. As a result, the higher *Mier3* levels found after carcinogen-exposure could be a reflection of early carcinogenesis, and possible reprogramming of the transcriptome of MECs preceding full cellular transformation.

I chose to evaluate *Mier3* expression at progressive pathological stages, as opposed to endpoint analysis in mammary tumors, as it is crucial to understand how the transcriptome changes during early carcinogenesis and disease progression to uncover prognostic or targetable molecular markers. There is a higher priority to identify a wide array of prognostic factors in an early disease state and prevent the development to a potentially lethal IDC altogether. Tumorigenesis in mammary glands is a multistep process that stems from the acquisition of genetic alterations that drive progressive transformation of MECs into malignant cells [474]. This can be studied within intermediate pathological stages, which in DMBA-induced mammary cancer progression is development of non-diseased mammary ducts to premalignant ADH, preinvasive DCIS, and to a full invasive carcinoma [483]. I evaluated *Mier3* expression based on (1) progression with time following carcinogen administration, and (2) tissue disease progression stages within each timepoint. I found significant increases in *Mier3* expression with increasing malignancy of

each disease morphology stage along the precursor-carcinoma sequence of ADH, DCIS, and IDC. This was found at all timepoints, with no differences in expression based on pathology when comparing across tissue collection timepoints. This indicates that it is not the time to progression that *Mier3* has a role in, for example a carcinoma that develops at 8 weeks post DMBA administration as compared to 15 weeks. Rather, *Mier3* expression increases between progressive tissue changes, indicating *Mier3* has a role in or is a product of the cellular transformations that occur throughout carcinogenic development to IDC.

In my progression analysis, I found the non-cancerous morphologies of non-diseased mammary ducts and ducts with usual hyperplasia possessed no differences in *Mier3* expression levels. Although usual hyperplasia encompasses a proliferation of MECs, this is not driven by carcinogenic transformation and does not contribute to breast cancer risk [467, 468], so the finding of similar *Mier3* levels to normal mammary ducts is not surprising. This indicates a shared role of *Mier3* in mammary ducts as in non-cancerous hyperplasias. In the early disease stage of ADH, I found a 1.6-fold increase in expression on top of the 2.2-fold increase in expression induced by DMBA-exposure. Following this large increase in *Mier3* expression, there was a progressive increase in expression within each of the final stages of DCIS and IDC. This suggests *Mier3* may have a role in tumor initiation and development. The initial increase in *Mier3* expression at the first pathological stage of ADH is noteworthy. These lesions are the earliest detectable changes to the breast epithelium and it is suggested that the molecular profile displays underlying susceptibility factors that drive further disease progression [484]. The increased *Mier3* expression between ADH and DCIS is possibly explained by the fact that DCIS typically displays much more frequent genetic alterations that drive tumorigenesis, even though these are

both preinvasive disease stages [471]. When evaluating disease pathologies based on overall percent of cells positive for *Mier3* expression rather than absolute quantification, I found a similar increase in *Mier3*-positive cells between non-diseased mammary ducts and ADH. This again suggests *Mier3* may have a role in pre-malignant cell transformation. However, there was no significant difference in the percent of cells positive for *Mier3* expression between ADH, DCIS, and IDC, even though the average transcripts per cell are increasing between these stages. This highlights that all cells in which *Mier3* has a potential tumorigenic role are present at the earliest disease stage. This complements what is known about global alterations in gene expression throughout mammary cancer progression, where significant transcriptional changes are observed during ADH and are maintained in DCIS and IDC [485]. This is thought to be due to the clonal relationship between these progression stages and suggests that the genes involved in carcinogenic growth and progressive potential are active at the earliest disease stage [464, 486]. Thus, increased *Mier3* expression in ADH may be due to neoplastic transformation and reflect a possible carcinogenic role of *Mier3* in early disease stages, which may be maintained and expanded along pathological progression to DCIS and IDC.

In my evaluation throughout rat mammary cancer progression, I found *Mier3* expression was most enhanced in mammary tumors. The correlation of increased *Mier3* expression with increasing malignancy of pathological stage, along with the greatest levels in IDC, suggests *Mier3* could have a role in tumor expansion or transition from pre-invasive to invasive growth. I also found variable expression levels of *Mier3* in IDCs, with some cells displaying such elevated levels that they are not discernable as distinct puncta, but rather appeared as a red smear across the cell nuclei. This may be a reflection of the

intra-tumoral heterogeneity that is common in DMBA-induced tumors and intrinsic to breast cancer [487, 488]. DMBA-induced mammary carcinomas consist of pleomorphic cells [487], with individual tumor cells differing in phenotypes and function, such as proliferation rates, angiogenic and metastatic potential, migration, induction of senescence, activation of signaling pathways, and response to anticancer drugs [488]. In addition to the neoplastic cells that constitute the tumor mass, there are networks of soluble factors, immune cells, vasculature cells, stromal cells, and extra-cellular matrix that together comprise the tumor microenvironment [489, 490]. These components can interact with the inherent genome instability of cancer cells and create diverse subpopulations within a tumor [490]. As a result, the extensive intra-tumoral variation in *Mier3* expression may have been a response to certain heterogeneous selective pressures that necessitate a role of *Mier3*, potentially in tumor growth, transition, invasion, or adaptation.

The overlap of the *Mcs1b* mammary cancer susceptibility gene locus and human breast cancer risk allele *5q11.2* nominates *Mier3/MIER3* as a breast cancer risk gene. In this study, I used a DMBA model of rat mammary cancer to investigate *Mier3* expression throughout a precursor-carcinoma sequence of disease development of ductal origin. This study shared similar goals as gene expression profiling; namely, to identify gene(s) that have the potential to be a molecular target in drug development or a molecular marker of disease diagnosis or prognosis [491, 492]. Importantly, this candidate gene approach was based on published experimental rationale that had established the phenotype of mammary cancer susceptibility was encompassed within this genetic region, as opposed to an uninformed, forward genetics approach.

A null result in this study, worthy of discussion, was the lack of *Mcs1b* genotypic differences in *Mier3* expression between my mammary cancer susceptible WF strain and my WF.COP *Mcs1b* resistant N3 strain. This could be a result of three *Mier3* transcript isoforms that arise from alternative splicing of exon 1 and an alternate 5' start site, generating transcripts with differing 5' ends and truncated N-termini of the translated protein products [256, 493]. However, these *Mier3* isoforms are not differentiated between using the *Mier3* RNAscope probe, which is designed to bind within nucleotides 2357-3614 of the 4758 base pair transcript, corresponding to the 3'UTR of *Mier3*. Previous analysis in our lab found lower expression of alternative isoforms in the resistant strain compared to the susceptible strain, but no differences with other *Mier3* transcripts (Samuelson lab, unpublished). Thus, the two genotypes may have variability in *Mier3* expression at the level of isoform differences specific to the mammary gland which could account for the difference in susceptibility. Additionally, this study only provides correlative information of *Mier3* expression quantification based on pathology stage. The purpose of this study was to identify mammary gland cell-specific expression of *Mier3* and changes with disease pathology in order to determine if this candidate breast cancer gene had a potential role in cancer risk and progression. The intent was to establish rationale to warrant in depth analysis of the function of MIER3 in breast cancer. Further mechanistic studies would need to be done to understand a molecular function of MIER3 in the context of breast cancer.

Genetic analysis has unveiled the complexity of breast disease, but there remains a lot to be uncovered about the molecular factors and changes that drive development and progression of premalignant breast lesions. Standard histopathology is limited in the ability to predict risk of disease progression, making it crucial to investigate the underlying

molecular biology. In this study, I detected a significant increase in *Mier3* expression in susceptible MECs with carcinogen exposure prior to development of any disease morphology, as well as increasing expression concurrent with increasing malignancy of pathological disease stage. This suggests a functional role of *Mier3* in disease initiation and tumorigenesis and elevates it as a potential biomarker or therapeutic target in early disease development. The highest *Mier3* levels were discovered in invasive carcinomas, which suggests an additional role in tumor growth. It is established that variation in transcriptional programs account for much of tumoral diversity and function [489]. Given the putative role of *Mier3* as a transcriptional repressor [494], elevated *Mier3* levels may reflect a potential role in MECs as a driver of breast carcinogenesis through transcriptional remodeling during disease initiation up through progression to invasive carcinomas. In summary, *Mier3* has a potential role in breast carcinogenesis and tumorigenesis, and may serve as a clinically meaningful target in disease progression.

CHAPTER VI

MIER3 EXPRESSION DURING MAMMARY GLAND DEVELOPMENT AND MAMMARY CARCINOMA SUSCEPTIBILITY WINDOWS

Introduction:

The developmental stages and morphology of the rat mammary gland closely matches that of the human breast [95, 96, 101, 117], and the DMBA-induced rat mammary cancer model induces similar pathology to human breast cancer [96, 219-221], making the rat a useful model in studying both human breast development and carcinogenesis. When studying the morphological and gene expression changes associated with mammary cancer progression, it is critical to examine these same changes that occur during mammary tissue development as many of the same factors that modify the developmental profile can contribute to carcinogenesis susceptibility later in life [95, 114, 117, 495, 496]. There are obvious similarities between the morphologies and growth pathways utilized during both development and carcinogenesis. The pubertal period displays rapid epithelial proliferation and expansion that is also necessary for tumor outgrowth, and the reproductive cycle undergoes controlled angiogenesis during pregnancy and lactation, as well as vascular regression and inflammation during involution that is also required to sustain tumor growth [114, 117, 132, 174, 495, 497]. Thus, the same processes involved in growth, proliferation, migration, invasion, and apoptosis to create a developed mammary gland are unfortunately

employed throughout tumorigenesis [117, 495, 498]. Unlike during carcinogenesis, the onset of these processes are tightly controlled and regulated during mammary gland development through interactions of hormones, growth factors, and signaling molecules with components of the extracellular matrix in the basement membrane as well as fibroblasts and adipocytes in the stroma [184, 495, 497, 499-501]. However, these same hormones and signaling factors in development can influence susceptibility to carcinogens at these time points and become dysregulated during breast cancer [95, 96, 502-504]. Overall, it is established that with any organ, it is crucial to obtain thorough knowledge of normal cellular and molecular mechanisms that guide development in order to understand initiation and progression of cancer [96, 133].

In addition to gross morphological changes that occur during mammary gland development, there exist cyclical changes to the human breast and rat mammary epithelium in response to ovarian hormone levels during the human menstrual cycle/rat estrus cycle [192, 199, 200]. Although tissue-specific changes are primarily confined to the ovaries and uterus, the mammary gland also undergoes cyclic stages of mammary epithelial cell (MEC) proliferation and regression due to ovarian hormone fluctuations, namely estrogen and progesterone [196, 199, 505-508]. Estrogen, specifically, has been associated with ductal elongation, and progesterone with ductal branching and lobulo-alveolar development [192, 196, 200, 509]. These morphological changes are more limited and minute, yet still mimic the broad tissue remodeling that occurs during pregnancy, lactation, and involution [200]. Epidemiological evidence indicates that the menstrual cycle increases the risk of female breast cancer, which may be attributable to endocrine control over susceptible MECs [510-512]. Rat estrus cycle-dependent stages of MEC proliferation and regression closely

matches changes in the human breast tissue during the menstrual cycle [193, 199, 513, 514], in which dysregulation of growth by ovarian hormone signaling could induce transformative events and contribute to mammary carcinogenesis [199].

The key stages of morphological changes in the mammary gland/breast in both the rat and human have been termed “windows of susceptibility” and represent timepoints when genetic risk factors and environmental perturbations result in increased lifetime risk of developing breast cancer [94-99]. Though the interaction of genetic and environmental factors can contribute to breast cancer risk at any age, these windows of susceptibility are time points in which a woman is more susceptible to cancer initiating events due to the rapid structural and molecular alterations taking place, which can play a role in breast cancer development later in life [94, 96, 98, 99]. Windows of susceptibility are comprised of developmental and reproductive time intervals in humans and rats, which include prenatal and pubertal development, pregnancy, lactation, involution, menopause, and premenopausal cyclical hormonal changes [97, 99]. Each time point has a distinct transcriptome, epigenetic changes, microenvironments, and signaling mechanisms unique to the biological function of the mammary gland/breast at that time, which can alter responses to environmental insults and heighten susceptibility to carcinogenesis [94, 96, 97, 100]. Clinical data has modeled this window of susceptibility phenomenon, where environmental exposures at these critical periods of susceptibility influence risk of future breast cancer development [97, 123, 515-518]. For example, exposure to ionizing radiation during childhood and adolescence increases the number of breast cancer cases but does not confer increased risk at older ages or after pregnancy [108, 519, 520]. This occurrence has also been demonstrated in rats using exposure to endocrine-disrupting carcinogens during

gestation, puberty, and pregnancy, which alters mammary gland development and results in increased tumor incidence following carcinogen administration [95, 112, 521-523]. Many of these environmental chemicals given to rats result in histological abnormalities and activate hormonal pathways that are postulated to enhance mammary gland susceptibility to carcinogenesis [95, 112, 517, 524, 525], but the molecular mechanisms responsible for effects on breast cancer risk remain largely unknown [99, 526]. To date, there appears to be only one report outlining mechanistically distinct susceptibility windows, where a noncoding locus in the rat was found to regulate downstream gene expression mediated by age-dependent chromatin looping and genotype-dependent DNA methylation [99]. There are over 180 genetic loci affecting breast cancer susceptibility that have been identified in human genome-wide association studies (GWAS) [99, 527], but their function throughout critical windows of susceptibility has not been assessed.

The rat model of the *Mcs1b* genetic susceptibility locus has nominated *Mier3*/*MIER3* as a breast cancer risk gene, which I confirmed as a breast cancer gene in my expression analysis throughout mammary cancer progression. Here, I sought to further our understanding of *Mier3* expression and ascertain a potential functional role during specific windows of susceptibility in the rat. This study was performed with the idea that a knowledge of *Mier3* expression at these time points could elucidate at what stages *Mier3* activity is potentially enhanced and responsible for increased risk of female breast cancer development. Analysis of *Mier3* expression during the stages of the estrus cycle was included to examine expression in relation to modulation of normal cyclical growth and regression of the mammary epithelium, which could contribute to carcinogenesis susceptibility. I report the highest *Mier3* levels in early mammary gland development

stages that are accompanied by extensive morphological changes, as well as fluctuations in expression correlating with the estrus cycle. These findings suggest *Mier3* may be hormonally regulated and possess a functional role in growth of MECs during embryonic through pubertal development. Combined with my previous finding of increased *Mier3* expression throughout mammary cancer progression, this indicates *Mier3* may serve a role in developmental mammary gland growth as well as a driver of mammary carcinogenesis at these crucial early windows of susceptibility.

Methods:

Animal husbandry

Rats were maintained in an AAALAC-approved facility on a 12 h light/dark cycle and provided LabDiet 5001 Rodent Diet (PMI® Nutrition International) and water ad libitum. Inbred Wistar Furth (WF/Hsd) females were obtained from Envigo. All animal protocols were approved by the University of Louisville Animal Care and Use Committee.

Congenetic Strains

The Wistar Furth (WF/NHsd) rat strain was used as a mammary cancer susceptible line. The Wistar Furth.Copenhagen (WF.COP) congenic rat strain N3 contains a segment of Copenhagen (COP/NHsd) on *RNO2* from markers D2Mgh2 to g2UL1-5 (Chr2:40171863 – 43756834, build RGSC 6.0/rn6) and is positive for the *Mcs1b* decreased susceptibility allele. The *Mcs1b* N3 strain at generation N16F41 – N16F42 was used as a mammary cancer resistance line and compared to the susceptible WF strain.

Mammary Gland Tissue Collection

Rats were euthanized by CO₂ asphyxiation. Abdominal-inguinal mammary glands from six females of both WF and WF.COP *Mcs1b* genotypes were resected and formalin-fixed and paraffin embedded (FFPE) at each post-natal developmental stage. Embryos were FFPE processed following head and limb removal. Limbs were kept for DNA extraction to sex embryos. Early development stages were taken at embryonic day 19, neonatal day 9, an early pubescent timepoint at 4 weeks of age, a pubescent timepoint at 8 weeks of age, and a nulliparous timepoint at 12 weeks of age. Reproductive cycle stages were taken at gestation day 19, lactation at 11 days post-parturition, involution 2 days following pup removal, and a parous timepoint at 24 weeks of age (4 weeks following litter weaning). Mammary glands used in analysis of the estrus cycle were also collected from parous animals at 24 weeks of age (Fig 39). Three animals were used per stage of the cycle.

Rat Embryo Sexing

Limbs removed from embryos were digested and DNA extracted. Gene specific oligonucleotide primers were used to PCR amplify portions of *Kdm5c* on the X-chromosome and *Kdm5d* on the Y-chromosome according to [528]. Primers for *Kdm5c* were F: 5'TTTGTACGACTAGGCCCCAC and R: 5'CCGCTGCCAAATTCTTTGG. Primers for *Kdm5d* were F: 5'TTGGGTGAGATGGCTGATTCC and R: 5'CCGCTGCCAAATTCTTTGG. Cycling conditions were as follows: initial denaturation 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 42 s, followed by a final extension for 7 min at 72°C

using an Applied Biosystems Veriti Fast Thermal Cycler. PCR products were resolved on a 1% LE agarose gel.

Estrus Cycle Staging

Vaginal cytology was used to classify combined WF and WF.COP *Mcs1b*-N3 females based on their stage in the estrus cycle. Vaginal cells were collected in PBS from rats using a plastic transfer pipet. Proestrus was classified by the appearance of small, rounded nucleated cells. Estrus appears as a majority cornified cells. Metestrus I appears as a late cornified stage with the presence of caseous fluid. At metestrus II there are leukocytes present with cornified cells. Diestrus is characterized by the presence of epithelial cells and leukocytes [529, 530].

Tissue Sectioning and Selection

Sectioning of FFPE blocks was done one day *prior* to a planned RNAscope assay, as mRNA remains more stable within paraffin blocks than tissue sections [460]. Mammary gland FFPE blocks were cut in 4um sections onto superfrost plus microscope slides (VWR) using a rotary microtome (Thermo HM 355S). Sections were assessed for integrity of mammary epithelial structures by H&E staining. Embryonic mammary buds from female embryos were captured by serial sectioning across the FFPE embryo body cavity and identifying the presence of mammary tissue by H&E staining.

Figure 39: Mammary gland collection timeline of windows of susceptibility during development and reproduction: Experimental stages of development, the reproductive cycle, and estrus cycle (top row) and corresponding timepoints (bottom row).

Hematoxylin and Eosin (H&E) staining of sectioned mammary glands:

Microscope slides were deparaffinized and rehydrated using 3 separate xylene washes followed by single washes each in 100% ethanol, 90% ethanol, 80% ethanol, and DI water. Tissues were stained with hematoxylin for 3 minutes, rinsed under running DI water for 3 minutes, soaked in bluing reagent for less than a minute, and again rinsed with DI water. Slides were dipped a few times in 100% ethanol to eliminate water before staining in eosin-Y for 30-45 seconds. Tissue was then dehydrated using washes of 90% ethanol, 100% ethanol, and 3 separate xylene washes. Samples were air dried and coverslipped using permount or similar mounting media.

RNA in situ hybridization

RNAscope was used to identify the spatiotemporal cell-specific expression of *Mier3* in rat mammary tissue according to manufacturer's directions (ACD Bio). Briefly, slides were deparaffinized in xylene and 100% ethanol, pretreated with an H₂O₂ block for 10 minutes, placed in target retrieval solution that was just under boiling (ACD Bio 322000) for 10 minutes, and treated with protease plus solution for 10 minutes (ACD Bio 322330). The target probe was added and slides were incubated in HybEZ oven (ACD Bio) at 40°C for 2 hours. Amplification steps were as follows using an RNAscope 2.5 HD red detection kit (ACD Bio 322360): Amp1 for 30 minutes at 40°C, Amp2 for 15 minutes at 40°C, Amp3 for 30 minutes at 40°C, Amp4 for 15 minutes at 40°C, Amp5 for 30 minutes at room temp, Amp6 for 15 minutes at room temp, and detection reagent for 10 minutes at room temp. Slides were counterstained with 1:1 Gill's hematoxylin and water. Vectashield

mounting media (Vector Laboratories H-1000) was added to protect staining. Slides were imaged at 40x on an Aperio ImageScope CS2.

Expression Quantification, Data Handling, and Statistical Analysis

Within images of tissue slides following the RNAscope assay, mammary duct, TEB, or lobular morphologies were outlined and selected for analysis using the HALO-ISH quantification software from Indica Labs. Mean probe per mammary epithelial cell and percent of *Mier3* positive mammary epithelial cells data were collected. Average *Mier3* transcript quantification values were transformed by taking the natural log. Percent positivity values were transformed by taking the arcsin of the square root of the percent values in decimal form. Transformed data were analyzed via one-way or two-way ANOVAs depending on the number of variables. Independent variables included timepoint, genotype, and morphology. All statistical analysis was done using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

Results:

To study mammary gland *Mier3* expression throughout mammary cancer windows of susceptibility, rat lifecycle was evaluated in three parts: (1) early mammary gland development, (2) the reproductive cycle, and (3) the estrus cycle. A mammary gland collection timeline of all experimental stages used in this study is outlined in Figure 39.

WF and WF.COP *Mcs1b*-N3 females have normal mammary gland morphology and express *Mier3* at each developmental timepoint

Early mammary gland development was analyzed in embryonic mammary buds at gestation day 19, and mammary glands at neonatal day 9, 4 weeks of age, 8 weeks of age, and 12 weeks of age. Figure 40 shows representative images of mammary gland gross morphologies and H&E stains at these developmental timepoints. Embryonic and neonatal timepoints were necessary to elucidate *Mier3* involvement at these critical structural periods of the primitive gland, while 4, 8, and 12 weeks of age were important to evaluate *Mier3* expression changes with onset of the estrous cycle and pubertal changes into an inactive adult mammary gland by 12 weeks of age. To study *Mier3* expression throughout the reproductive cycle, mammary glands were collected from pregnant, lactating, involuting, and parous rats. The previous 12 week timepoint dually functioned as a nulliparous counterpart to the parous stage. Figure 41 shows representative images of the mammary gland gross morphologies and H&E stains at these reproductive timepoints. Nulliparous and parous timepoints were selected to determine baseline *Mier3* expression in inactive mammary glands, whether virgin or fully differentiated. The other timepoints were critical to evaluate *Mier3* expression changes with tissue remodeling and the different functional roles of active, secretory mammary glands during pregnancy, lactation, and involution. Figures 40 and 41 also display that mammary glands collected at each developmental stage appear morphologically as expected, indicating our rat strains used have normal mammary gland development and are appropriate to assay with RNAscope.

The sensitivity of the RNAscope assay was tested using *Mier3* as a moderately expressing gene, and control probes *RNA Polymerase II Subunit A (Polr2a)*

Figure 40: Mammary Gland Appearance and Histology during Development: Top row – gross morphology of the intact mammary gland at each time point, except for a full embryo at E19 shown. Middle row – Low power view of H&E stained mammary glands to show overall histology. Bottom row – High power view of H&E stained mammary glands to show morphological features.

The embryonic mammary bud extends from the body cavity and is captured by sectioning across (A). Primitive epithelial structures can be observed at late embryonic development. The postnatal mammary fat pad grows ventro-laterally along the skin. During neonatal development (9 days) a clustering of epithelial ducts can be detected within a mostly stromal mammary gland component. At puberty (4 and 8 weeks) fully developed bi-layered epithelial ducts (B) are apparent throughout the stromal matrix. At the ends of ducts are proliferative terminal end buds (TEBs) (C) that drive ductal extension and branching. At 12 weeks of age the epithelial tree has fully expanded and developed within the mammary fat pad, and is marked by a loss of the majority of TEB structures.

Figure 41: Mammary Gland Appearance and Histology during the Reproduction

Cycle: Top row – gross morphology of the intact mammary gland at each time point.

Middle row – Low power view of H&E stained mammary glands to show overall histology.

Bottom row – High power view of H&E stained mammary glands to show morphological features.

The fully developed virgin (nulliparous) mammary gland is mainly comprised of branching epithelial ducts (A) interspersed within connective tissue stroma and surrounded by adipose tissue. Smaller ductules (B) and alveolar buds (B) are also apparent as tubular clusters. During pregnancy, alveolar buds differentiate into milk-secreting lobular structures (C) that encompass the majority of the fat pad. The ductal tree regresses and becomes a minimal structure at this time. The lactating mammary gland consists of actively secreting lobular structures (D) that have attained maximal volume in the mammary fat pad. Loss of suckling results in retention of milk and fat globules within lobules (E) that initiates involution. Tissue remodeling, mainly controlled apoptosis of lobules and re-establishment of the ductal tree, brings the mammary gland back to a quiescent state. The parous gland resembles the nulliparous gland except for some retained differentiated lobular structures (F).

as a highly expressed gene and bacterial *4-hydroxy-tetrahydrodipicolinate reductase* (*DapB*) as a negative control. Each probe functioned as expected based on differences in density of puncta between *Mier3* and *Polr2a*, and lack of puncta with *DapB*. Probes were assayed in tissue used to study early development (Fig 42) and the reproductive cycle (Fig 43).

***Mier3* expression is greatly enhanced in mammary ducts and terminal end buds during embryonic through pubertal development, but significantly drops off at adulthood**

Mier3 expression was first analyzed in mammary epithelial structures during the early stages of mammary gland development in WF and WF.COP *Mcs1b*-N3 females. Primitive embryonic mammary tissue was unable to be excised in the same manner as mammary glands post-birth, so embryonic body cavities were serial sectioned across and stained to find tissue. Since this is an extremely laborious process, tissue was analyzed from combined genotypes. This late stage of embryonic development possessed the greatest levels of *Mier3* (Fig 44). There was significantly greater *Mier3* expression in WF mammary ducts at the neonatal timepoint of 9 days old, quantified as either mean probe per MEC ($p=0.0380$ unpaired two-tailed t test) or percent of MECs positive for *Mier3* ($p=0.0025$) (Fig 45).

During puberty, terminal end bud (TEB) morphologies first appear as a prominent, proliferative epithelial structure of the pubescent mammary gland. As such, there was separate analysis of ductal and TEB morphologies in early stage puberty (4 weeks old) and standard puberty (8 weeks old). There was no significant genotypic difference during early

Figure 42: RNAscope assay probes in rat mammary glands throughout development.

From top to bottom are representative 40x images of embryonic mammary buds, primitive ducts at 9 days, a mix of ductule and terminal end bud (TEB) morphology at 4 weeks, TEBs at 8 weeks, and ducts at 12 weeks. The RNAscope assay probed for *Mier3* as a moderately expressed gene (left column), *Polr2a* as a highly expressed gene (middle column) and bacterial *DapB* as a negative control (right column).

Figure 43: RNAscope assay probes in rat mammary glands during the reproductive cycle. From top to bottom are 40x representative images of mammary glands during pregnancy, lactation, and involution. At each timepoint ductal (left panel) and lobular (right panel) morphologies are shown. The RNAscope assay probed for *Mier3* as a moderately expressed gene (left column), *Polr2a* as a highly expressed gene (middle column) and bacterial *DapB* as a negative control (right column).

Figure 44: *Mier3* expression in MECs of rat embryonic mammary buds: (A) 40x mammary bud histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary buds. (C) Quantification of MECs positive for *Mier3* expression. N=6 embryonic mammary buds, N=26 total fields of view.

Figure 45: *Mier3* expression in MECs of rat mammary glands at 9 days of age: (A) 40x mammary duct histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts in the WF vs N3 strain. There was a significant difference based on genotype using an unpaired two-tailed t test ($p < 0.05$). (C) Quantification of MECs positive for *Mier3* expression. There was a significant difference based on genotype using an unpaired two-tailed t test ($p < 0.01$). N=6 mammary glands per genotype, minimally n=14 fields of view per genotype.

puberty at 4 weeks of age, but there was a morphology difference when quantifying by mean *Mier3* probe per MEC ($p=0.9892$ genotype, $p<0.0001$ morphology, two-way ANOVA) and percent of MECs positive for *Mier3* expression ($p=0.8399$ genotype, $p<0.0001$ morphology, two-way ANOVA). In both strains, TEBs displayed greater *Mier3* levels than mammary ducts by mean *Mier3* probe quantification per MEC ($p<0.0001$ Tukeys *post hoc*) and overall MECs positive for *Mier3* ($p=0.0003$) (Fig 46). The main effect at this timepoint was morphology as there was initially a genotypic difference during the standard pubertal timepoint when quantifying by mean probe per MEC, but this was lost in *post hoc* analysis ($p=0.0095$ genotype, $p=0.0020$ morphology, two-way ANOVA). When quantifying by percent of *Mier3* positive MECs, there were significant differences during puberty at 8 weeks of age ($p=0.0154$ genotype, $p<0.0001$ morphology, two-way ANOVA). There was a significant genotypic difference in *Mier3* expression within ductal morphologies ($p=0.03$ Tukeys *post hoc*), but none in comparing TEBs. There was also significantly greater expression in TEBs in the WF strain ($p=0.03$) and WF.COP *Mcs1b*-N3 strain ($p=0.0002$) (Fig 47).

A final developmental timepoint of 12 weeks of age was next evaluated, as this is breeding age in the rat and is representative of fully developed, adult mammary glands. Pubescent TEB structures are minimal at the timepoint, so only mammary ducts were evaluated. *Mier3* expression was greatly reduced at this stage compared to previous timepoints. There was no significant difference between genotypes based on mean *Mier3* probe analysis ($p=0.4089$ unpaired two-tailed t test) but there was a greater percent of *Mier3*-positive MECs in the WF strain ($p=0.0179$) (Fig 48). Total quantification of *Mier3* expression at all developmental timepoints is given in Table 24.

Figure 46: *Mier3* expression in MECs of rat mammary glands at 4 weeks of age:

(A) 40x images of a mammary duct (left) and TEB (right) from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts and TEBs in the WF vs N3 strain. There was no difference based on genotype, but a significant difference based on morphology (2-way ANOVA, Tukeys *post hoc* $p < 0.0001$). (C) Quantification of MECs positive for *Mier3* expression in mammary ducts and TEBs. There was no difference based on genotype, but a significant difference based on morphology (2-way ANOVA, Tukeys *post hoc* $p = 0.0003$). N=6 mammary glands per genotype, minimally n=20 fields of view per genotype per morphology.

Figure 47: *Mier3* expression in MECs of rat mammary glands at 8 weeks of age:

(A) 40x images of a mammary duct (left) and TEB (right) from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts and TEBs in the WF vs N3 strain. There was no significant difference based on genotype or morphology. N=6 per genotype. (C) Quantification of MECs positive for *Mier3* expression in mammary ducts and TEBs. There was a significant difference based on genotype in ducts (2-way ANOVA, Tukeys *post hoc* $p=0.01$) and between morphologies ($p<0.0001$). N=6 mammary glands per genotype, minimally n=12 fields of view per genotype per morphology.

Figure 48: *Mier3* expression in MECs of rat mammary glands at 12 weeks of age:

(A) 40x mammary duct histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts in the WF vs N3 strain. There was no significant difference based on genotype. (C) Quantification of MECs positive for *Mier3* expression in mammary ducts. There was a significant difference based on genotype (unpaired two-tailed t test $p=0.02$). $N=6$ per mammary glands genotype, minimally $n=20$ fields of view per genotype.

Table 24. *Mier3* expression in WF/NHsd and WF.COP *Mcs1b*-N3 mammary epithelial structures throughout early development

Genotype	Age	Stage	Morphology	Mean probe per cell (SD)	% of positive cells (SD)
WF/NHsd and WF.COP <i>Mcs1b</i> -N3	E19	Embryonic	Mammary bud	5.27 (0.75)	74.0 (4.6)
WF/NHsd	P9	Neonatal	Duct	2.83 (0.54)	51.2 (3.8)
WF.COP <i>Mcs1b</i> -N3	P9	Neonatal	Duct	2.48 (0.74)	57.5 (10.2)
WF/NHsd	4wks	Early puberty	Duct	3.35 (0.39)	67.9 (3.6)
WF/NHsd	4wks	Early puberty	Terminal end bud	4.08 (0.58)	73.7 (5.2)
WF.COP <i>Mcs1b</i> -N3	4wks	Early puberty	Duct	3.28 (0.53)	68.5 (5.0)
WF.COP <i>Mcs1b</i> -N3	4wks	Early puberty	Terminal end bud	4.17 (0.50)	73.6 (3.7)
WF/NHsd	8wks	Puberty	Duct	3.25 (0.82)	63.4 (6.6)
WF/NHsd	8wks	Puberty	Terminal end bud	3.75 (1.06)	68.1 (6.5)
WF.COP <i>Mcs1b</i> -N3	8wks	Puberty	Duct	2.66 (0.24)	57.3 (4.4)
WF.COP <i>Mcs1b</i> -N3	8wks	Puberty	Terminal end bud	3.28 (0.56)	67.2 (5.4)
WF/NHsd	12wks	Adult	Duct	1.08 (0.33)	43.9 (9.4)
WF.COP <i>Mcs1b</i> -N3	12wks	Adult	Duct	0.97 (0.18)	38.0 (5.0)

N=6 mammary glands per genotype per timepoint, E19=6 mammary buds total

***Mier3* expression significantly increases during growth phases of the reproductive cycle and has variation between ductal vs lobular morphologies**

The next phase of *Mier3* expression analysis throughout windows of susceptibility in the rat mammary gland was broken up into reproductive development. The adult timepoint of 12 weeks of age in the previous early development phase was also included as the start to the reproductive cycle, since this nulliparous stage provides baseline *Mier3* expression information in undifferentiated and inactive mammary glands. During late pregnancy, lobuloalveolar structures appear as the predominant morphology in meeting the demands of lactation and persist throughout the cycle. As such, *Mier3* probe quantification was performed separately in mammary duct and lobular morphologies at each stage analyzed.

At 19 days gestation, there was significantly greater *Mier3* expression compared to the nulliparous stage. In evaluating this pregnancy timepoint, there were significant differences based on genotype and morphology when quantifying mean *Mier3* probe per MEC ($p=0.0107$ genotype, $p<0.0001$ morphology, two-way ANOVA) and overall *Mier3*-positive MECs ($p=0.00006$ genotype, $p<0.0001$ morphology, two-way ANOVA). However, the effect of genotype was lost in *post hoc* analysis in mean quantification and only persisted in lobular morphologies in percent positivity analysis ($p=0.04$ Tukeys *post hoc*). Mammary ducts possessed significantly greater *Mier3* levels than lobules ($p=0.02$), as well as greater percent of *Mier3*-positive MECs ($p=0.002$ WF, $p=0.003$ N3) (Fig 49).

The next stage of the reproductive cycle selected was 11 days post-parturition, which represents a timepoint of peak mammary gland lactation. Quantification by mean *Mier3* probe per MEC and *Mier3*-positive MECs gave significant differences based on

Figure 49: *Mier3* expression in MECs of rat mammary glands during pregnancy:

(A) 40x images of a mammary duct (left) and lobule (right) from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software.

(B) Quantification of mean *Mier3* expression per MEC in mammary ducts and lobules in the WF vs N3 strain. There was no difference based on genotype, but a significant difference between morphologies (2-way ANOVA, Tukeys *post hoc* $p < 0.0001$).

(C) Quantification of MECs positive for *Mier3* expression in mammary ducts and lobules.

There was a significant difference based on genotype in lobules (2-way ANOVA, Tukeys *post hoc* $p = 0.0006$) and between morphologies ($p < 0.0001$). $N = 6$ mammary glands per genotype, minimally $n = 20$ fields of view per genotype per morphology.

Figure 50: *Mier3* expression in MECs of rat mammary glands during lactation:

(A) 40x images of a mammary duct (left) and lobule (right) from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts and lobules in the WF vs N3 strain. There was a significant difference between genotype in ducts (2-way ANOVA, Tukeys *post hoc* $p < 0.0001$) and between morphologies ($p < 0.0001$). (C) Quantification of MECs positive for *Mier3* expression in mammary ducts and lobules. There was a significant difference based on genotype (2-way ANOVA, Tukeys *post hoc* $p < 0.0001$) and morphology ($p < 0.0001$). N=6 mammary glands per genotype, minimally n=17 fields of view per genotype per morphology.

genotype and morphology ($p < 0.0001$ two-way ANOVA). Analyzing by mean *Mier3* probe quantification, there were greater levels in ductal structures in the N3 strain ($p < 0.0001$ Tukeys). *Post hoc* analysis of percent of *Mier3*-positive MECs yielded significantly greater levels in the N3 strain ($p < 0.0001$ duct, $p = 0.03$ lobule) as well as greater ductal positivity in both strains ($p = 0.04$ WF, $p < 0.0001$ N3) (Fig 50).

To evaluate the process of involution, the timepoint of 2 days following cessation of lactation via pup removal was selected, as this represents entry into the irreversible phase of involution. Quantification via mean *Mier3* probe per MEC was significant ($p = 0.0007$ genotype, $p < 0.0001$ morphology, two-way ANOVA), but *post hoc analysis* showed the genotypic significance only in lobular structures ($p = 0.02$ Tukey's *post hoc*). Analysis by percents of *Mier3*-positive MECs yielded only a morphological difference ($p = 0.1391$ genotype, $p < 0.0001$ morphology, two-way ANOVA). Differing from the phases of active growth in pregnancy and lactation, involution displayed greater *Mier3* levels in lobular structures as opposed to ductal (Fig 51).

A final parous timepoint was selected for studying *Mier3* mammary gland expression at 4 weeks following weaning of pups. This represents a time where involution has remodeled the mammary gland back into an inactive, yet now fully differentiated, gland. Although mammary ducts are once again the predominant structures, quantification was separated based on morphology since some lobules remain present. *Mier3* levels fell back down and were similar to that found in nulliparous mammary glands. Analysis by mean *Mier3* probe quantification and percent of *Mier3*-positive MECs gave no significant differences based on genotype or morphology (two-way ANOVA) (Fig 52). Total quantification of *Mier3* expression at all reproductive timepoints is given in Table 25.

Figure 51: *Mier3* expression in MECs of rat mammary glands during involution:

(A) 40x images of a mammary duct (left) and lobule (right) from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts and lobules in the WF vs N3 strain. There was a significant difference between genotype (2-way ANOVA, Tukeys *post hoc* $p=0.0007$) and morphologies ($p<0.0001$). (C) Quantification of MECs positive for *Mier3* expression in mammary ducts and lobules. There was a significant difference in morphologies (2-way ANOVA, Tukeys *post hoc* $p<0.0001$). N=6 mammary glands per genotype, minimally n=17 fields of view per genotype per morphology.

Figure 52: *Mier3* expression in MECs of parous rat mammary glands: (A) 40x images of a mammary duct (left) and lobule (right) from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. The bottom row is the red probe and nuclei markup from the HALO-ISH module software. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts and lobules in the WF vs N3 strain. (C) Quantification of MECs positive for *Mier3* expression in mammary ducts and lobules. For both graphs there was no significant difference based on genotype or morphology. N=6 mammary glands per genotype, minimally n=18 fields of view per genotype per morphology.

Table 25. *Mier3* expression in WF/NHsd and WF.COP *Mcs1b*-N3 mammary epithelial structures throughout reproductive development

Genotype	Stage	Morphology	Mean probe per cell (SD)	%of positive cells (SD)
WF/NHsd	Nulliparous*	Duct	1.08 (0.33)	43.9 (9.4)
WF.COP <i>Mcs1b</i> -N3	Nulliparous*	Duct	0.97 (0.18)	38.0 (5.0)
WF/NHsd	Pregnancy	Duct	1.99 (0.48)	54.2 (6.4)
WF/NHsd	Pregnancy	Lobule	1.50 (0.48)	47.9 (7.8)
WF.COP <i>Mcs1b</i> -N3	Pregnancy	Duct	1.61 (0.32)	49.9 (5.8)
WF.COP <i>Mcs1b</i> -N3	Pregnancy	Lobule	1.27 (0.21)	43.0 (3.3)
WF/NHsd	Lactation	Duct	1.18 (0.26)	41.8 (7.2)
WF/NHsd	Lactation	Lobule	1.13 (0.35)	36.1 (5.8)
WF.COP <i>Mcs1b</i> -N3	Lactation	Duct	1.95 (0.50)	58.7 (8.5)
WF.COP <i>Mcs1b</i> -N3	Lactation	Lobule	1.17 (0.29)	42.0 (5.6)
WF/NHsd	Involution	Duct	1.18 (0.27)	40.3 (5.7)
WF/NHsd	Involution	Lobule	1.73 (0.27)	46.8 (4.1)
WF.COP <i>Mcs1b</i> -N3	Involution	Duct	1.04 (0.18)	41.6 (6.7)
WF.COP <i>Mcs1b</i> -N3	Involution	Lobule	1.48 (0.17)	48.9 (3.6)
WF/NHsd	Parous	Duct	1.22 (0.34)	43.1 (7.2)
WF/NHsd	Parous	Lobule	1.23 (0.34)	42.7 (6.6)
WF.COP <i>Mcs1b</i> -N3	Parous	Duct	1.06 (0.48)	37.3 (10.2)
WF.COP <i>Mcs1b</i> -N3	Parous	Lobule	1.03 (0.41)	38.4 (10.0)

* Same values as adult stages in Table 23

N=6 mammary glands per genotype per timepoint

Summary: *Mier3* expression is highest at embryonic through pubertal stages within developmental susceptibility windows in the rat mammary gland

Comparing across stages of development in this study, there was a significant difference based on timepoint but no effect of genotype ($p=0.0647$ genotype, $p<0.0001$ timepoint, two-way ANOVA). WF and WF.COP *Mcs1b*-N3 genotype data were combined for ductal morphologies at each stage, since TEB and lobular morphologies are not present across all developmental timepoints. There were significant expression differences across mammary gland development stages evaluated ($p<0.0001$ one-way ANOVA) (Fig 53). *Mier3* expression was highest during the embryonic stage, and significantly differed from every other stage ($p<0.0001$ Tukey's *post hoc*). The next highest timepoints of 9 days of age and 4 weeks of age significantly different from each other ($p=0.0033$ Tukey's *post hoc*), but not from the timepoint of 8 weeks of age. Expression dropped to the lowest levels at 12 weeks of age/nulliparous glands, during involution, and in parous glands. These timepoints did not significantly differ from each other. There was a significant increase in *Mier3* expression during pregnancy and lactation as compared to the other stages of the reproductive cycle ($p<0.0001$ Tukey's *post hoc*), but this was also significantly lower than the early development stages.

The rat mammary epithelium displays cyclical changes in *Mier3* expression, with the greatest levels correlated with the hormonal stages of the estrus cycle

In the concluding analysis of windows of susceptibility timepoints in rat mammary glands, *Mier3* expression was evaluated at each stage of the rat estrus cycle since cyclical

Figure 53: Ductal Expression of *Mier3* in MECs of rat mammary glands during development: Quantification of mean *Mier3* probe per MEC in combined WF and WF.COP *Mcs1b*-N3 mammary duct morphology at each mammary gland development stage. Different lowercase letters indicate timepoints with significantly different average transcript levels ($p < 0.001$). A significant one-way ANOVA ($p < 0.0001$) followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.00 Software. Minimally $N=26$ total fields of view per timepoint.

changes to the mammary gland can alter susceptibility. Staging was determined based on vaginal cytology, which is informative of the cyclical growth and regression of the mammary epithelium. Combined WF and WF.COP *Mcs1b*-N3 genotypes were used since these tissues were collected at the same age as the parous timepoint which showed no genotypic differences. Quantification was based on stage and morphology, with ductal and lobular morphology separated at each timepoint (Fig 54) (Table 26). Quantification based on mean *Mier3* probe per MEC yielded no difference between ductal and lobular morphologies, but showed a significant difference between stages of the cycle in (p=0.1095 morphology, p<0.0001 stage, two-way ANOVA). Using Tukey's *post hoc* analysis, the stages of proestrus, estrus, and metestrus I were grouped as significantly higher than the other stages, but not different from each other. Expression at metestrus II was significantly lower than this collective group (p=0.001 Tukey's *post hoc*), followed by diestrus which possessed the lowest levels (p=0.0001 Tukey's *post hoc*). Quantification by overall MECs positive for *Mier3* gave a significant difference based on morphology and stage (p=0.0268 morphology, p<0.0001 stage, two-way ANOVA). In the *post hoc* analysis, proestrus and estrus were found to significantly differ from each other (p=0.0004 Tukey's *post hoc*) but neither differed from metestrus I. Similar to mean probe quantification, metestrus II was significantly lower than these stages (p=0.001 Tukey's *post hoc*) and diestrus was lowest overall (p=0.0001 Tukey's *post hoc*). Using both quantification methods, the greatest *Mier3* levels corresponded with the most hormonally-active stages of the estrus cycle.

A

B

***Mier3* expression in MECs of rat mammary glands by stages of the estrus cycle**

C

***Mier3* positive MECs in rat mammary glands at stages of the estrus cycle**

Figure 54: *Mier3* expression in rat mammary glands based on stage of the rat estrus cycle: (A) 40x mammary gland histologic images from RNAscope assays probing for *Mier3* show red puncta (*Mier3* transcripts) counterstained with hematoxylin. From top to bottom shows a representative mammary duct at each consecutive stage of the estrus cycle – proestrus, estrus, metestrus I, metestrus II, and diestrus. (B) Quantification of mean *Mier3* expression per MEC in mammary ducts and lobules in the WF strain. There was no significant difference based on morphology, but a significant difference based on stage of the cycle (2-way ANOVA, Tukeys *post hoc* $p < 0.0001$). (C) Quantification of MECs positive for *Mier3* expression in mammary ducts and lobules. There was a significant difference based on morphology (2-way ANOVA, Tukeys *post hoc* $p = 0.03$) and stage of the cycle (Tukeys *post hoc* $p < 0.0001$). Different lowercase letters indicate significant Tukey's multiple comparisons tests ($p < 0.001$). N=3 mammary glands per estrus cycle stage, minimally n=20 fields of view per genotype per morphology.

Table 26. *Mier3* expression in combined WF/NHsd and WF.COP *Mcs1b*-N3 mammary epithelial structures throughout the rat estrus cycle

Estrus Cycle Stage	Morphology	Mean probe per cell (SD)	% of positive cells (SD)
Proestrus	Duct	1.44 (0.44)	36.8 (5.2)
Proestrus	Lobule	1.44 (0.35)	38.9 (4.4)
Estrus	Duct	1.66 (0.37)	43.2 (4.3)
Estrus	Lobule	1.77 (0.47)	45.2 (6.3)
Metestrus I	Duct	1.56 (0.35)	40.4 (5.9)
Metestrus I	Lobule	1.55 (0.27)	40.3 (5.3)
Metestrus II	Duct	1.28 (0.23)	36.8 (4.7)
Metestrus II	Lobule	1.30 (0.20)	37.3 (3.8)
Diestrus	Duct	1.03 (0.32)	31.6 (6.2)
Diestrus	Lobule	1.19 (0.36)	34.2 (6.2)

N=3 mammary glands per stage

Discussion:

In my analysis of mammary gland *Mier3* expression during windows of susceptibility, I quantified expression in mammary epithelial cells (MECs) based on average transcripts per cell, as well as overall percent of MECs positive for *Mier3* expression. At each stage assayed, a change in average probe number per cell positively correlated with a change in percent positivity. This indicates that *Mier3* expression is modulated not within a set subpopulation of MECs, but within the MEC population as a whole. As a result, *Mier3* may serve a universal function in MECs at each developmental stage.

I found a positive correlation between *Mier3* mammary gland expression and known ovarian hormone signaling levels based on stages of the rat estrus cycle. *Mier3* expression was highest during proestrus, estrus, and metestrus I, which encompass a window of peak estrogen surging and ovulation, followed by a rise in progesterone levels, and a peak in progesterone during metestrus I [199]. *Mier3* levels began to decline at metestrus II, corresponding to when ovarian hormone signaling significantly drops off [199, 202]. I found the lowest levels of *Mier3* during diestrus, which is characterized as a non-hormonal phase of the estrus cycle [200]. These findings are significant because even though these are inactive mammary glands, *Mier3* expression still seems to be modulated in relation to hormonal cycling. It is established that the mammary epithelium undergoes cyclical proliferation and regression due to endocrine control over MECs, namely estrogen and progesterone [199, 505, 506]. However, there are differing publications of MEC changes based on the rat estrus cycle stage, with proestrus, metestrus, and diestrus all reportedly possessing peak MEC proliferation levels [196, 199, 203]. There remains a lot

to be uncovered regarding how the estrus cycle effects the mammary epithelium, but the high expression of *Mier3* in MECs and cyclical changes in *Mier3* expression suggests *Mier3* may have a role in this process. The correlation of highest *Mier3* expression during the hormonally-dominated stages of the estrus cycle also suggests *Mier3* may be hormonally regulated.

I found the highest *Mier3* expression levels ranging from embryonic development through pubertal development, with a drastic decrease in expression in the adult mammary gland. This loss in expression in the adult gland indicates a diminished role of *Mier3* once the mammary gland has achieved pubertal development and entered into an inactive stage. High *Mier3* expression during embryonic and pubertal development corresponds with significant periods of allometric growth in the mammary gland, where in rats, the gland grows at 3.5-5 times the rate of the rest of the body [531]. There was a small dip in expression during neonatal development which corresponds to a period of isometric growth, where the rate of mammary gland growth is kept in rate with regular body growth [532]. Allometric mammary gland growth during this developmental period is largely in response to estrogen signaling, which is responsible for the expansive ductal growth that occurs, especially during puberty [531, 533]. In fact, deletion of $ER\alpha$ or its pharmacological inhibition hinders ductal growth, which is restored with exogenous estrogen treatment [507, 531, 534, 535]. There are other endocrine cues driving morphological development, including progesterone and prolactin, whose mammogenic effects include induction of ductal side branching and alveolar bud formation [536]. Additionally, growth hormone and insulin-like growth factor-I influence ductal elongation [535]. Thus, different levels of mammogenic hormones establish numerous conditions for

stages of morphogenesis of the mammary gland [535, 536], but the exact roles and levels of hormones and growth factors in rat mammary gland development is unclear. Estrogen is known to bind to its receptor and stimulate production of other growth factors [536], which may include *Mier3* given its association with cancerous and developmental growth. However, ER does not appear in MECs in either rodents or humans during fetal development [536-538]. I determined from this study of the estrus cycle that *Mier3* appears to act in concert with ovarian hormone fluctuations and is possibly hormonally regulated. Yet, given its highest involvement during fetal development, which lacks ER, *Mier3* may be stimulated by alternative hormonal pathways, in addition to or independent of estrogen.

Increased expression of *Mier3* was found during the pubertal phase of mammary gland development, including early initiation of puberty at 4 weeks of age. At each pubescent time point analyzed, terminal end bud (TEB) morphology had greater *Mier3* levels as compared to ductal structures. This finding correlates with my discovery of increasing *Mier3* expression along a ductal mammary cancer progression model, as these proliferative structures contain vulnerable precursor cells that are commonly held to be the initiation sites of ductal carcinogenesis [102, 209, 464]. TEBs are clusters of undifferentiated epithelial cells at the distal ends of primary ducts, which continuously expand with each round of estrus cycling and drive proliferation of the mammary epithelium [104, 114, 167]. The finding that the most proliferative mammary gland structure possesses the highest *Mier3* expression levels adds further support that *Mier3* has an important functional role in MEC growth.

I report significantly higher *Mier3* expression in MECs during late gestation and lactation stages as compared to nulliparous glands. However, the overall average *Mier3*

transcripts per MEC at these time points do not match the high expression levels found during embryonic, neonatal, and pubertal development. This is likely due to the drastic architectural and hormonal differences that exist in the mammary gland between pubertal and reproductive development. During pregnancy, there is a great increase in alveolar bud formation and differentiation into alveoli, which cluster and form functional secretory lobules. This lobuloalveolar growth continues post-parturition [94, 133]. Lobuloalveolar formation relies on differing signaling mechanisms than ductal morphogenesis. For example, progesterone and prolactin are largely responsible for driving alveologensis and differentiation [94]. As a result, the differences in *Mier3* expression found between pre-pubescent/pubescent growth and reproductive growth may be a result of the different hormonal milieu, which creates a reduced role for *Mier3* or differentially modulates *Mier3* expression. There is also a known decrease in proliferative activity corresponding with increasing differentiation of mammary structures, so reduced *Mier3* expression may be attributable to the state of differentiation of MECs. I believe the increased levels of *Mier3* I found during pregnancy and lactation is correlated with its presumed role in MEC growth; however, the substantially lower levels as compared to early development is likely due to the differences in types of MEC growth and differentiation that occur during reproductive development.

In addition to analyzing overall *Mier3* expression during pregnancy and lactation, I quantified expression based on lobular or ductal morphology, and found greater expression in MECs of ducts as compared to lobules. This may be due to the differing functions of luminal epithelial cells based on morphology; serving a secretory role in lobules as opposed to solely a conduit for the movement of milk in ducts. As a result, these

cells may respond to growth stimuli or signaling molecules differently. *Mier3* is expressed at lower levels in lobules, and late gestation and lactation are mostly lobular growth, which could indicate why this growth period doesn't recapitulate the high levels recorded in early developmental growth. Conversely, during involution we found greater lobular expression of *Mier3* as compared to ductal. I took mammary glands at the start of the irreversible phase of involution, which is marked by massive rates of apoptosis and removal of the secretory epithelium [94]. Overall *Mier3* expression during the period of involution was significantly lower than pregnancy and lactation, and no different than the quiescent nulliparous and parous stages. Thus, the greater lobular expression may not necessarily indicate a role of *Mier3*, but instead point to a lack of a role of *Mier3* in the inactive ductal structures at this stage.

I found no significant differences in *Mier3* expression between nulliparous mammary glands at 12 weeks of age and parous glands at 24 weeks. In the parous mammary gland there still remains some alveoli, but overall it is morphologically similar to the virgin gland [94]. The major difference between these stages of mammary gland development is at the molecular level, as parity results in the achievement of complete differentiation and a distinct transcriptional profile from the undifferentiated, nulliparous counterpart [539, 540]. Pregnancy also drives terminal differentiation of the proliferative MECs most vulnerable to carcinogenesis, resulting in a protective effect from breast cancer risk [541]. My results indicate that *Mier3* expression, although specific to MECs, is not contingent upon or driven by the state of differentiation of these cells. Additionally, since *Mier3* expression is unchanged going to a differentiated stage, it is not part of the transcriptional reprogramming of MECs and likely doesn't have a mechanistic role in

altered disease susceptibility post-parturition. The nulliparous and parous stages displayed the lowest *Mier3* levels in our study of windows of susceptibility, so the lack of difference in expression may also be attributable to the quiescent state of the mammary glands, not the degree of differentiation.

A null result in this study was no genotype differences for the most part between my mammary cancer susceptible WF strain and my WF.COP *Mcs1b* resistant N3 strain. It is possible that this is due to the existence of three *Mier3* alternative splice variants [256, 493]. Populations of MECs from the opposing genotypes may exhibit variable expression and functionality of the differing splice variants but have no differences in total *Mier3* expression overall. The *Mier3* RNAscope probe mix possesses 20 probe pairs that are designed to bind within nucleotides 2357-3614 of the 4758 base pair transcript, so no variation in alternative splice forms would be captured in this assay. There was statistical indication of genotypic differences at 9 days of age and during lactation; however, the lack of a difference in genotype at any other stage puts this finding under scrutiny. I only used 6 animals per genotype at each stage of development so we could be statistically underpowered in our analysis. The purpose of this study was to determine what stage of development *Mier3* potentially has a role in, with the intent of further evaluation at interesting stages. I found that early developmental stages encompassing embryonic, neonatal and pubertal development displayed the highest *Mier3* expression, making these stages the best candidates for re-evaluation and further analysis into the role of *Mier3*.

In summary, I found high *Mier3* expression levels at stages of mammary gland development that are characterized by extreme morphological changes that make the gland lactation-competent. Morphological re-structuring of the mammary gland is driven by

transcriptional reprogramming, as well as hormonal signaling and growth factor stimulation. The high expression levels I report at these stages complement the putative transcriptional repressive function of MIER3 [494], that could have a mechanistic role in promoting tissue evolution. Based on my results, I propose a functional role of Mier3 in the growth or differentiation of MECs during embryonic through pubertal mammary gland development. I also suggest that Mier3 is hormonally regulated. Given its identified role throughout mammary cancer progression, it is especially relevant that *Mier3* is highly expressed in the exact cell type and stages most vulnerable to cancer initiating events, in what is most often a hormonally-driven disease. Thus, I conclude that in addition to a developmental growth function, MIER3 could serve as a potential driver of breast carcinogenesis at these developmental time windows of susceptibility.

CHAPTER VII

MIER3 PROTEIN INTERACTIONS SUGGEST A FUNCTIONAL ROLE IN A TRANSCRIPTIONAL CO-REPRESSOR COMPLEX

Introduction:

The MIER family members share structural features in common with known transcriptional co-repressors, including the ELM2 and SANT domains [261, 262]. The ELM2 (Egl-27 and MTA1 homology 2) domain was initially identified in the MTA1 protein that is a component of the NuRD chromatin regulatory complex [542] and has since been shown to recruit trichostatin A-sensitive (class I) histone deacetylase (HDAC) proteins that lead to changes in chromatin structure and transcriptional repression [543, 544]. It has been demonstrated that the ELM2 domain of MIER1 α and β is responsible for recruiting and binding HDAC 1 and 2, providing one mechanism of transcriptional repression [543]. The ELM2 domain of MIER1 is also necessary for binding the histone acetyltransferase (HAT) Creb-binding protein (CBP), which provides another mechanism of transcriptional repression as this interaction inhibits the histone acetyltransferase activity of CBP [270]. The vast majority of ELM2 containing proteins also possess a SANT domain immediately downstream, indicating a functional or structural relationship between these motifs. SANT domains are highly conserved sequences of around 50 amino acids that were first identified in the transcriptional factors SWI3, ADA2, N-CoR, and TFIIB, generating

the acronym “SANT” [545]. They have since been found in the subunits of many chromatin-remodeling complexes [546]. The SANT domain is characterized by tandem repeats of three α -helices that are arranged in a helix–turn–helix motif, similar to the DNA-binding domain of Myb proteins; however, the SANT domain does not bind DNA or contribute to transcriptional repression in the same manner [547, 548]. The SANT domain is thought to instead serve a chromatin remodeling function by facilitating interactions with histones [545]. In MIER1, the SANT domain has been demonstrated to bind transcription factor specificity protein 1 (Sp1) at the MIER1 promoter, displacing it from its cognate sites, and repressing its own transcription as well as other Sp-1 regulated genes [263]. This represents an HDAC-independent manner of transcriptional repression by MIER1. The protein binding domains of the other MIER family members may also facilitate interactions with chromatin modifying proteins, similar to established co-repressors.

MIER2 and MIER3 are hypothesized to possess transcriptional repressive roles similar to MIER1 due to sequence homology and shared ELM2/SANT domains, but investigations into their individual mechanisms are still in their infancy. It has been shown that all MIER family members associate in HDAC complexes, but never with each other or as dimers [264]. They also do not appear in complexes with other corepressor complexes such as CoREST (co-repressor of RE1-silencing transcription factor), NuRD (nucleosome remodeling deacetylase), Sin3, or NCoR (nuclear receptor corepressor), suggesting each MIER forms independent HDAC-containing complexes that are functionally distinct from each other [264]. HDAC recruitment also appears to be cell-line dependent. For example, in HEK293 cells, MIER1 and 2 associates with HDAC1 and 2, but not MIER3. In MCF-7 and HeLa cells, MIER1 associates with both HDACs, MIER2 only with HDAC1, and

MIER3 with neither [264]. In CEM-T cells all MIER family members were found in HDAC1 and 2-containing complexes [267]. Finally, in K562 cells MIER2 and 3 were found to only associate with HDAC2 [549]. This variability in MIER-HDAC complexes highlights not only the differences between family members, and likely underlying functionality, but also the cell-line dependent manner of the composition of the complexes, indicating environment and tissue type create alternative roles for the MIER proteins. Despite shared similarities with respect to their domains and regions of high homology, these proteins are also divergent, especially at their C-termini, suggesting distinct molecular mechanisms among them in transcriptional regulation. MIER1 and 2 also share more similarities between them than with MIER3, indicating MIER3 especially has diverse functional roles. For example, the presence of MIER1 and 2 have been reported in chromodomain Y like (CDYL) chromatin repressive complexes, and HDAC1/2 association with this complex is improved by MIER1/2 interaction [550-552]. However, MIER3 is not found within this complex. MIER1/2, but not MIER3, are also enriched at RE1-silencing transcription factor (REST) target genes [552]. Even the shared domains of the MIER family may be functionally distinct. MIER1/2 are predicted to contain coiled-coiled motifs in their C-terminal regions of the ELM2 domain while MIER3 possess an LXXLL motif at this region instead [552]. MIER3 also contains an additional LXXLL motif near its N-terminus, while MIER1 α only contains one at the C-terminus, and MIER2 does not possess any. Thus, the MIER family members appear to participate in unique complexes from each other, which may serve overlapping functions as transcriptional repressors, but there are likely distinct effects on downstream gene regulation.

The established and predicted transcriptional repressive roles of the MIER family as members of co-repressor complexes also indicates dysregulation of these genes could have an important impact on cancer biology and disease development. Epigenetic regulation of gene expression allows for cell-specific transcriptional programs that impart differing cell functions despite having the same genetic information [553]. However, perturbations to these processes can lead to aberrant gene expression which contribute to disease initiation and tumor progression [554, 555]. There exist multiple layers of transcriptional regulation, which include direct methylation of DNA, covalent modifications to histone proteins which contribute to chromatin structure, and nucleosome remodeling which represents higher order chromatin organization [556]. Co-repressor complexes are often recruited and bound to DNA by transcription factors, where the enzymatic activities of chromatin-modifying protein subunits work in a combinatorial fashion to recognize, remove, and place epigenetic marks that result in chromatin condensation [557, 558]. Differences in subunit composition create unique specificities for certain genomic loci [559]. Altered levels of chromatin co-repressor proteins disrupts the balance of transcriptional activation and repression that results in oncogenesis [560, 561]. In breast cancer specifically, there is global hypomethylation accompanied by formation of repressive chromatin domains and gene silencing early in disease development [562]. MIER1 has an established role in breast cancer as member of a co-repressor complex, which may contribute to these disease-specific repressive chromatin domains. Although it is unknown whether MIER3 exists in a co-repressor complex, it has an association with human breast cancer as a candidate rat mammary cancer susceptibility gene [256]. In this study, I sought to identify the protein-protein interactors of MIER3 within a hormone

receptor positive breast cancer cell line in order to determine if MIER3 exists in a co-repressor complex and what the subunit composition is in a breast disease state. I also approached this study with a specific hypothesis that MIER3 interacts with a nuclear hormone receptor via its LXXLL sequence motifs that directs certain chromatin interactions, since the possession of two motifs as compared the single motif in MIER1 α could elucidate an impotent functional difference between these MIER proteins.

Methods:

Plasmid Construction and Site Directed Mutagenesis

Homo sapiens MIER3 ORF (NCBI/GenBank ref NM_152622.3) from pooled human breast total RNA (#AM6952, Life Technologies) was cloned into pEGFP-C1 vector at EcoRI and KpnI sites. Primer sequences for cDNA amplification were 5'-CGGAATTCTATGGCGGAGGCTTCTTTTGGAAGT-3' and 5'-CGGGGTACCCTCAGAGTGTAGGGCCGCGTGC-3'. MIER3 LXXLL sequence motifs were mutated to LXXAA using a QuikChange Multi Site-Directed Mutagenesis kit (Agilent 200515). Primer sequences for LEDLL mutagenesis was 5'-GGAAGGAACCATGCCTCTAGAAGATGCAGCGGCATTCTATGGCTATGAAC and LYELL mutagenesis was 5'-AAGGGACAATGAACAGGCATTATATGAAGCTGCCAAGTGTAACCACAATATAAAGGAAGC-3'. Mutations were verified using primer sequences for cDNA amplification, and Sanger sequenced with an ABI PRISM 7700 Sequence Detection System using the LEDLL sequencing primer 5'-CCCACTGCTGAGATGTTGGT-3' and LYELL sequencing primer 5'-ACTTTGGTGTCTCTGATGTGGT-3'.

Cell culture and Transfection (standard conditions)

T47D (#HTB-133) cell line was purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 with 10% FBS, 1% antibiotic-antimycotic, and 0.2 U/mL bovine insulin. Cell authentication was guaranteed by ATCC and morphology was confirmed under a phase-contrast light microscope. T47D cells were plated at 5×10^6 cells per 10cm dish. Using Lipfectamine 3000 transfection reagent (ThermoFisher L3000015) and following the manufacturers protocol, 20 plates were transfected with 28 μ g plasmid in opti-MEM with 1% antibiotic-antimycotic for each plasmid- pEGFP-C1, pEGFP-C1-MIER3, and pEGFP-C1-MIER3-LXXAA.

Cell culture and Transfection (serum starvation and stimulation)

Following adherence, cells were serum starved in RPMI 1640 without L-glutamine and phenol red with 1% charcoal/dextran treated FBS and 1% antibiotic-antimycotic. Using Lipfectamine 3000 transfection reagent (ThermoFisher L3000015) and following the manufacturers protocol, 20 plates were transfected with 28 μ g plasmid in opti-MEM with 1% anti-anti for each plasmid- pEGFP-C1, pEGFP-C1-MIER3, and pEGFP-C1-MIER3-LXXAA. Cells were re-stimulated 6 hours post transfection with the addition of RPMI 1640 with 40% FBS and 1% anti-anti to the transfection mixture, for a total of 20% serum.

Evaluation of transfection efficiency

At 24 hours post transfection, efficiency was estimated in 3 plates each of cells transfected with pEGP-C1 or pEGFP-C1-MIER3. This was done using T47D and HEK293

cell lines. Cells positive for GFP fluorescence were identified using an EVOS digital inverted fluorescent microscope. Representative images were taken at 10x.

Transfection of pEGP-C1 and pEGFP-C1-MIER3 was repeated in 6 plates of T47D cells each. At 8 hours post transfection, 3 plates per plasmid were treated with 10 μ M MG132 [563]. At 24 hours post transfection, the number of GFP-positive cells were counted in minimally seven separate 10x fields of view per plasmid using an EVOS digital inverted fluorescent microscope. Representative images were taken at 10x. This experiment was repeated and positive cells counted every 2 hours up to 24 hours post transfection, with a final assessment at 48 hours.

Cell Lysis and Affinity Purification of GFP-interacting proteins

At 24 hours following transfection, cells were harvested on ice in lysis buffer with 2.5 mg/mL DSP (40 mM HEPES pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1x protease/phosphatase cocktail inhibitors, and dithiobis succinimidyl propionate (DSP, Pierce) 200 mg/ml in DMSO). Total cell lysate was collected into a single tube per transfected plasmid- pEGFP-C1, pEGFP-C1-MIER3, and pEGFP-C1-MIER3-LXXAA. Cell lysates were incubated on ice for 30 minutes, cross linking quenched with 1M Tris-HCl pH 7.4 in a ration of 1:4, and incubated for a further 30 minutes on ice. Lysates were clarified by centrifugation at 4500xg for 40 min at 4°C and filtered through SpinX columns (Corning). Lysates were pre-cleared by incubating with 50 μ L packed sepharose beads (Sigma Aldrich) for 1 hour at 4°C on a rotating wheel. GFP-TRAP beads (ChromoTek) were equilibrated in lysis buffer and 30 μ L of pelleted beads added to each lysate. Lysates were spun on a rotating wheel at 4°C for 4 hours. Beads were washed 4x for 1 hour each

at 4°C in lysis buffer supplemented with 0.5 M NaCl, and washed 1x in 50 mM Tris-HCl, pH 7.5, 0.1mM EGTA. Beads were reconstituted in 80 µL 1x LDS sample buffer with 0.1M DTT, incubated 1 hour at 37°C, and boiled for 5 minutes at 95°C. Denatured proteins were eluted through SpinX columns.

In-Gel Protein Digestion and Extraction:

Protein elution was run on an 4-20% gradient polyacrylamide gel (BioRad 4561094) at 100V for 1 hour then stained with Coomassie blue to visualize bands. Bands were cut into roughly 1 mm³ plugs, place in 1.5 mL tubes, 10 µL of 100 mM triethylammonium bicarbonate (TEA-BC) added, and tubes incubate at room temperature for 15 minutes. Tubes were incubated for another 15 minutes following addition of 15 µL acetonitrile (ACN). Solvent was removed by SpeedVac for about five minutes. To the dry tubes, 20 µL of 20 mM DTT/100mM TEA-BC was added and tubes incubated at 56°C for 45 minutes. DTT solution was removed, 20 µL of 55 mM iodoacetamide/100mM TEA-BC was added, and tubes incubated at room temperature for 30 minutes in the dark. Iodoacetamide solution was removed, 200 µL of 50 mM TEA-BC was added, and tubes incubated at room temperature for 15 minutes. Tubes were incubated for another 15 minutes following addition of 200 µL ACN. Solvent was removed and 3 µL of digestion buffer (20 ng/µL modified trypsin in 50 mM TEA-BC) was added to gel pieces. After about 10 minutes gel pieces swelled and 50 mM TEA-BC was added to a final volume of 25 µL. Tubes were incubated at 37°C overnight in a shaker.

To extract peptides, 25 µL of LC-MS grade water was added to the tubes, followed by 100 µL of 1:2 5% v/v formic acid in water:acetonitrile, and tubes incubated at room

temperature for 15 minutes in a shaker. Liquid surrounding the gel pieces was transferred to a clean tube and solvent removed in a SpeedVac. Protein was re-dissolved in 20 μ L 2% v/v acetonitrile / 0.1% v/v formic acid. Sample was filtered through a 0.45 μ m regenerated cellulose syringe filter (Thermo #F2504-7) to remove any gel material, and 2-20 μ L injected for LC-MS analysis

1-Dimensional Reverse Phase Liquid Chromatography (LC)-MS/MS:

Approximately 400 ng of fractionated peptides were analyzed by 1-dimensional nano-LC on a Thermo Ultimate 3000 HPLC system directly eluting into a Thermo Q-Exactive-HF mass spectrometer. Peptides were separated on a 15 cm in-house packed tip reverse phase 3.6 μ m XB-C-18 resin (Phenomenex, Aeris C18) column and direct eluted at 200 nl/min into the MS. Briefly, a reverse phase gradient from 5 minutes to 90 minutes was applied with 0% solvent B (80% acetonitrile, 0.1% formic acid) to 45% solvent B for each of the 18 fractions in sequence. Peptides were eluted directly into a Thermo Q-Exactive-HF at a spray voltage of 1.65 kV, 250°C and spectra were acquired in data dependent top 15 ion mode.

Computational analysis:

Spectra were deconvoluted by Proteome Discoverer v2.2 and the built-in SEQUEST analysis program used to map peptides to the *in silico* Uniprot human reviewed database. Peptide false discovery rate was held at <1%. LC-MS/MS analysis performed following band excision gave a more comprehensive list of peptides than analysis of direct protein elution, so identifications of less than 1 unique peptide were not considered in

downstream analysis and were removed from the dataset of these experiments. SEQUEST compares projected theoretical spectra to the observed tandem mass spectrum using cross correlation analysis [564]. Resulting SEQUEST HT scores were used to evaluate the strongest candidate peptides. Proteins in the MIER3-GFP interaction list were compared to the GFP trap interaction list and removed if deemed non-specific. Protein lists were submitted to the CRAPome (a Contaminant Repository for Affinity Purification Mass Spectrometry Data) (www.crapome.org), which was used to further identify background contaminants and score interacting proteins ranked by confidence [565]. Protein IDs were filtered out for highly non-specific interactions as predicted from literature reports aggregated in the CRAPome database. CRAPome performs a fold change (FC) calculation on the ratio of average normalized spectral counts in bait purifications versus negative controls. The more stringent secondary FC-B score was accepted as the final stringency criteria in parsing out the strongest protein interactors.

A final list of the strongest protein interactors in which to focus further analysis on was created by accepting the top 25% of protein IDs across the three stringency criteria of SEQUEST HT score, number of peptide fragments detected, and CRAPome FC-B score. Proteins that did not appear in at least 2 of these 3 lists were excluded.

This list was subjected to gene ontology analysis through the Gene Ontology Consortium (www.geneontology.org) to analyze interacting proteins based on shared biological processes and molecular function [566]. A Fishers exact test with stringent Bonferroni correction was used and analysis type was a PANTHER Overrepresentation Test (GO Ontology database DOI: 10.5281/zenodo.4081749 Released 2020-10-09). The

output with each topic is the number of proteins from the input list, the expected number of proteins, the resulting fold enrichment, and the associated Bonferroni P-value.

Toppgene, a portal for gene list enrichment analysis and candidate gene prioritization, was used to further functionally analyze the list of strongest nuclear protein interactors (www.toppgene.cchmc.org) [567]. Specifically in this portal, ToppFun was used for functional enrichment of the input protein list based on gene expression, protein domains and interactions, and ontologies. Co-expression analysis within ToppFun found breast cancer microarrays as the top hits [444, 568, 569] which output gene lists and Bonferroni P-values.

Results:

MIER3 overexpression is not permissible and the protein product is likely rapidly degraded

The overall goal of this analysis was to identify MIER3 protein-protein interactions, with the specific intention of isolating a nuclear hormone receptor, to determine if MIER3 is participating in a chromatin repressive complex similar to MIER1. To first test the transfection efficiency of the MIER3 plasmid, T47D breast cancer cells were transfected with either pEGFP-C1 empty vector or pEGFP-MIER3-C1 and resulting GFP fluorescence analyzed 24 hours later. Cells transfected with empty vector displayed high levels of fluorescence while pEGFP-MIER3-C1 transfected cells showed very low levels of fluorescence at only 0-2 cells fluorescing in 10x fields of view (Figure 55). To determine if this low transfection efficiency resulted from the MIER3 insert, this same transfection procedure was repeated in HEK293 cells. This cell line has an established high transfection

efficiency and dependable protein translation and processing [570, 571]. HEK293 cells displayed even greater GFP fluorescence intensity with the empty vector control than T47D cells, which was expected from this highly transfectable cell line. There again were only a few fluorescent cells observed in each 10x field of view with cells transfected with pEGFP-MIER3-C1 (Figure 55). This same disparity in fluorescence between plasmids indicates that transfection is successful in both cell lines, but MIER3 overexpression may be signaling rapid degradation of its protein product.

Proteasome inhibitor treatment increases MIER3 expression but does not recapitulate levels of the empty vector control

To superficially test if the hypothesis of rapid MIER3 protein degradation might hold some validity, T47D cells were treated with the proteasome inhibitor MG132 at 8 hours following transfection to halt possible MIER3 protein degradation. A dose of 10 μ M MG132 was selected as this has been shown to effectively inhibit protein degradation *in vitro* in carcinoma cell lines, including breast carcinoma lines MCF-7 and T47D [572-574]. Fluorescence was then analyzed at 24 hours post-transfection. As before, T47D cells transfected with eGFP-C1 displayed significantly greater numbers of fluorescent cells as compared to pEGFP-MIER3-C1, but there was no effect of MG132 treatment. Cells transfected with pEGFP-MIER3-C1 displayed significantly greater numbers of fluorescent cells when treated with MG132 as compared to untreated (Figure 56). This experiment was repeated, and fluorescence monitoring extended to every 2 hours following MG132 treatment. Results were consistent between experiments. Cells transfected with pEGFP-C1 displayed no difference in fluorescence when treated with MG132. Cells transfected with

Figure 55: Cells transfected with pEGFP-MIER3-C1 display low levels of fluorescence. Shown are representative images of cells at 10x magnification on an EVOS digital inverted fluorescent microscope at 24 hours post-transfection. **A, C:** T47D cells (top row) and HEK293 cells (bottom row) transfected with pEGFP-C1 empty vector display high levels of fluorescence. **B, D:** Fluorescence levels drop substantially in both cell lines transfected with pEGFP-MIER3-C1.

A

B

Figure 56: pEGFP-MIER3-C1 transfected cells display increased fluorescence with proteasome inhibitor treatment. Shown are representative images of T47D cells at 10x magnification on an EVOS digital inverted fluorescent microscope at 24 hours post-transfection alongside the quantification of GFP positive cells from differing fields of view. **A, C:** Cells transfected with pEGFP-MIER3-C1 display greater numbers of GFP positive cells with 10 μ M MG132 treatment as compared to control. **B, D:** 16 hours of MG132 treatment does not have an effect in pEGFP-C1 transfected cells. Data were analyzed by a 2-tailed Mann-Whitney test, **** $p < 0.0001$

Figure 57: pEGFP-MIER3-C1 transfected cells display increased fluorescence over a 48 hour time course with proteasome inhibitor treatment. Shown is the quantification of GFP positive T47D cells at 10x magnification and differing fields of view at every 2 hours following MG132 treatment. **A:** pEGFP-MIER3-C1 transfected cells display greater numbers of GFP positive cells with MG132 treatment as compared to control. This is greatly enhanced by 14 hours post-treatment. **B:** MG132 treatment does not have an effect in pEGFP-C1 transfected cells over time. Fluorescence data for each plasmid transfection with and without MG132 treatment were separately analyzed by 2-tailed Mann-Whitney tests, **** $p < 0.0001$.

pEGFP-MIER3-C1 and treated with MG132 displayed significantly greater numbers of GFP positive cells beginning at 20 hours post-transfection (Figure 57). In all groups, levels drop off at the final timepoint of 48 hours as cell death begins to take place. Although GFP positive cells as a result of pEGFP-MIER3-C1 transfection don't recapitulate the numbers from the GFP empty vector, the significant increase in numbers from proteasome inhibition lends support to the hypothesis that the MIER3 protein may be signaled for degradation as a result of overexpression. These experiments determined that pEGFP-MIER3-C1 transfection was occurring, albeit resulting in lower GFP-MIER3 protein levels than desired.

Affinity purification of GFP-MIER3 followed by LC-MS/MS recapitulates reported HDAC1/2 and BAHD1 interactions, with BAHD1 specific to the LXXLL motifs

Towards the goal of isolating and confirming an interaction with a nuclear hormone receptor, site-directed mutagenesis was performed on the pEGFP-MIER3-C1 plasmid to mutate the LXXLL sequence motifs of MIER3 to LXXAA. This particular mutation has been established as sufficient to disrupt protein-protein binding at LXXLL motifs [575-577]. This plasmid contains isoform 1 of human MIER3, which possess an LEDLL sequence at amino acids 66-70 and a downstream LYELL sequence within the ELM2 domain at amino acids 251-255. The resulting plasmid population from site-directed mutagenesis was used to transform competent cells. Colonies were selected, the MIER3 insert PCR amplified, and PCR products digested by the restriction enzyme TseI to screen for LXXLL mutations (Figure 58). Plasmid was purified from colonies indicating single and double mutations from TseI digestion and were Sanger sequenced to verify LXXLL to

Figure 58: Screening of transformed colonies for LXXLL mutations of MIER3.

MIER3 contains two LXXLL sequence motifs, LEDLL and LYELL. Shown is a 1.4% agarose gel of the MIER3 PCR product from *E. Coli* colonies transformed with site-directed mutagenesis treated pEGFP-MIER3-C1 that was digested with TseI. Expected bands from digestion of non-mutated MIER3 is 812 and 846 bp; single LEDAA mutant is 211, 601, and 846 bp; single LYEAA mutant is 46, 766, and 845 bp; and double LEDAA/LYEAA mutant is 46, 211, 555, and 846 bp.

Figure 59: The LXXLL sequence motifs of MIER3 were successfully mutated to LXXAA. Shown are electropherograms from Sanger sequencing of pEGFP-MIER3-C1 plasmids that contain non-mutated sequences for LEDLL and LYELL (top row) or mutated sequences for LEDAA and LYEAA (bottom row).

LXXAA mutations (Figure 59). This yielded MIER3 plasmid with LXXAA mutations to compare protein interactions specific to the LXXLL motifs of the non-mutated pEGFP-MIER3-C1 plasmid.

The previous analysis of the transfection efficiency of pEGFP-MIER3-C1 showed a low amount of GFP-MIER3 protein being translated. To overcome this low protein concentration, a total of 60 dishes (10cm) of T47D cells were plated, with 20 dishes per plasmid – GFP empty vector, GFP-MIER3, and GFP-MIER3-LXXAA. Whole cell lysate was collected 24 hours post transfection and GFP-trap beads used to affinity purify GFP tags following protein crosslinking. GFP, GFP-MIER3, and GFP-MIER3-LXXAA ran on an SDS-PAGE gel at their expected molecular weights of 27 kD for GFP and a combined 88 kD for GFP-MIER3 (Figure 60). The total elution obtained from affinity purification was applied to LC-MS/MS analysis. This experiment was repeated once more, and the peptide IDs identified by mass spectroscopy appearing in both pull downs were accepted as “real” interactors. Table 27 provides a list of these IDs unique to MIER3 and MIER3-LXXAA following removal of peptides also appearing with the GFP empty vector and common GFP contaminants identified by CRAPome analysis. Figure 61 illustrates the number of overlapping peptides identified. Most notable in this list are HDAC1, HDAC2, and BAHD1, all which have been previously reported [264, 578]. HDAC1/2 have been demonstrated to bind to the ELM2 domain of the MIER family of proteins, and as a result also appear with the MIER3-LXXAA. However, the BAHD1 interaction disappears with the mutant, indicating this interaction is likely specific to the LXXLL sequence motifs. From these experiments we did not detect the hypothesized interaction with a nuclear hormone receptor.

Figure 60: MIER3 proteins appear at the correct molecular weight following affinity purification of GFP. Coomassie blue staining of an SDS-PAGE gel run after a GFP-trap experiment using pEGFP-C1, pEGFP-MIER3-C1, and pEGFP-MIER3-C1-LXXAA. GFP runs at the expected molecular weight of 27 kD, while GFP-MIER3 with and without mutations runs at the expected weight of 88 kD.

Table 27. Mass spectrometry peptide IDs from total elutions of GFP-trap pulldowns unique to MIER3 and MIER3-LXXAA

MIER3			MIER3-LXXLL Mutant		
Gene	Description	Sequest HT	Gene	Description	Sequest HT
MIER3	Mesoderm induction early response protein 3	536.21	FASN	Fatty acid synthase	12.93
LMNB1	Lamin-B1	43.58	MIER3	Mesoderm induction early response protein 3	13.98
ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	39.78	PPIA	Peptidyl-prolyl cis-trans isomerase A	28.79
STAT1	Signal transducer and activator of transcription 1-alpha/beta	44.25	CLTC	Clathrin heavy chain 1	6.91
ATAD3	ATPase family AAA domain-containing protein 3A	40.16	GSR	Glutathione reductase	2.28
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	29.99	PRDX2	Peroxiredoxin-2	6.66
BAHD1	Bromo adjacent homology domain-containing 1 protein	75.8	FLNB	Isoform 8 of Filamin-B	3.61
KPNA2	Importin subunit alpha-1	39.96	ARF3	ADP-ribosylation factor 3	2.41
IDH2	Isocitrate dehydrogenase, mitochondrial	51.32	RAB1C	Putative Ras-related protein Rab-1C	2.37
PHGDH	D-3-phosphoglycerate dehydrogenase	36.06	CFL1	Cofilin-1	7.32
MAGED2	Melanoma-associated antigen D2	38.65	PDIA6	Isoform 2 of Protein disulfide-isomerase A6	4.65
TCP1	T-complex protein 1 subunit alpha	29.7	HSD17B10	3-hydroxyacyl-CoA dehydrogenase type-2	4.24
AIFM1	Apoptosis-inducing factor 1	21.26	ALYREF	THO complex subunit 4	2.11
OAS2	2'-5'-oligoadenylate synthase 2	32.12	IQGAP1	Ras GTPase-activating-like protein IQGAP1	2.06
SQOR	Sulfide:quinone oxidoreductase	45.65	AHNAK	Neuroblast differentiation-associated protein AHNAK	1.86
ATP5F1B	ATP synthase subunit beta	46.66	CAPZA1	F-actin-capping protein subunit alpha-1	0
HDAC2	Histone deacetylase 2	90.93	RAB5C	Ras-related protein Rab-5C	0
TRIP13	Pachytene checkpoint protein 2 homolog	26.15	STIP1	Isoform 2 of Stress-induced-phosphoprotein 1	0
GALK1	Galactokinase	36.4	CRKL	Crk-like protein	0
PRKDC	DNA-dependent protein kinase catalytic subunit	31.17	VCP	Transitional endoplasmic reticulum ATPase	0
FAR1	Fatty acyl-CoA reductase 1	31.17	EPPK1	Epiplakin	0
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	19.84	PFKP	ATP-dependent 6-phosphofructokinase	0
UNC45A	Protein unc-45 homolog A	16.56	HDAC1	Histone deacetylase 1	3.67
DDX21	Nucleolar RNA helicase 2	16.27	HDAC2	Histone deacetylase 2	1.66
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	19.94	STAT1	Signal transducer and activator of transcription 1-alpha/beta	2.14
SLC25A6	ADP/ATP translocase 3	43.51	KPNB1	Importin subunit beta-1	0
RARS	Arginine--tRNA ligase	16.43	FN1	Isoform 15 of Fibronectin	2.96
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	19.94	NPEPPS	Puromycin-sensitive aminopeptidase	2.85
NSF	Vesicle-fusing ATPase	32.45	PARK7	Protein/nucleic acid deglycase DJ-1	2.76
BAG3	BAG family molecular chaperone regulator 3	30.2	FARSA	Phenylalanine--tRNA ligase alpha subunit	2.69
HLA-B	HLA class I histocompatibility antigen, B-37	25.37	CRIP2	Cysteine-rich protein 2	2.59

EHHADH	Peroxisomal bifunctional enzyme	25.37	CALML5	Calmodulin-like protein 5	2.5
FOXK1	Forkhead box protein K1	19.55	LYZ	Lysozyme C	2.45
ARAF	Serine/threonine-protein kinase A-Raf	19.46	PPA1	Inorganic pyrophosphatase	2.39
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	18.41	ABHD14B	Protein ABHD14B	2.34
EHD4	EH domain-containing protein 4	16.52	TGM1	Protein-glutamine gamma-glutamyltransferase K	2.26
ACSL3	Long-chain-fatty-acid--CoA ligase 3	13.31	PSMA8	Proteasome subunit alpha-type 8	2.22
FARSA	Phenylalanine--tRNA ligase alpha subunit	27.74	SFXN2	Sideroflexin-2	2.21
SLC25A3	Phosphate carrier protein	30.7	LGALS1	Galectin-1	1.88
MCCC2	Methylcrotonoyl-CoA carboxylase beta chain	16.94	CDK1	Cyclin-dependent kinase 1	1.87
SLC25A4	ADP/ATP translocase 1	40.82	TBR1	T-box brain protein 1	1.82
CKAP4	Cytoskeleton-associated protein 4	16.16	CORO1B	Coronin-1B	1.75
ABCF3	ATP-binding cassette sub-family F member 3	19.81	ECM1	Isoform 4 of Extracellular matrix protein 1	1.73
FXR1	Fragile X mental retardation syndrome-related protein 1	25.21	ATP5F1B	ATP synthase subunit beta	1.71
CCT4	T-complex protein 1 subunit delta	18.12	EPRS	Bifunctional glutamate/proline--tRNA ligase	1.7
DARS	Aspartate--tRNA ligase	17.35	PTBP1	Isoform 3 of Polypyrimidine tract-binding protein 1	1.7
ACSL1	Long-chain-fatty-acid--CoA ligase 1	13.27	ATP5PO	ATP synthase subunit O	1.63
HADHB	Trifunctional enzyme subunit beta	15.19	IDH2	Isocitrate dehydrogenase	0
IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	14.75	ZNF354B	Zinc finger protein 354B	0
ESRP2	Epithelial splicing regulatory protein 2	15.43	FARSB	Phenylalanine--tRNA ligase beta subunit	0
ACAD11	Acyl-CoA dehydrogenase family member 11	25.92	PRKDC	DNA-dependent protein kinase catalytic subunit	0
SUCLA2	Succinate--CoA ligase subunit beta,	13.15	IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	0
ERLIN1	Erlin-1	16.72	MCM3	Isoform 2 of DNA replication licensing factor MCM3	0
DTX3L	E3 ubiquitin-protein ligase DTX3L	13.04	TRIM28	Transcription intermediary factor 1-beta	0
MARK2	Serine/threonine-protein kinase MARK2	16.2	EMSY	BRCA2-interacting transcriptional repressor EMSY	0
GARS	Glycine--tRNA ligase	13.56	SLC25A13	Calcium-binding mitochondrial carrier protein Aralar2	0
DNAJA2	DnaJ homolog subfamily A member 2	11.92	PCK2	Phosphoenolpyruvate carboxykinase	0
QARS	Glutamine--tRNA ligase	12.7	PPP2CB	Serine/threonine-protein phosphatase 2A catalytic subunit	0
ALDH3A2	Isoform 2 of Fatty aldehyde dehydrogenase	10.27	P3H4	Endoplasmic reticulum protein SC65	0
SDHA	Succinate dehydrogenase flavoprotein subunit	11.4	SRF3	Serine/arginine-rich splicing factor 3	0
CLTC	Clathrin heavy chain 1	9.14	PFKL	ATP-dependent 6-phosphofructokinase	0

KHSRP	Far upstream element-binding protein 2	8.05	GFPT1	Glutamine--fructose-6-phosphate aminotransferase 1	0
DDOST	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	8.36	OGA	Isoform 3 of Protein O-GlcNAcase	0
MTA2	Metastasis-associated protein MTA2	7.89	ARL3	ADP-ribosylation factor-like protein 3	0
ATP5PO	ATP synthase subunit O	7.53	THBS1	Thrombospondin-1	0
SEC23B	Protein transport protein Sec23B	7.08	CTSD	Cathepsin D	0
NSUN2	tRNA (cytosine(34)-C(5))-methyltransferase	8.01	DDX6	Probable ATP-dependent RNA helicase DDX6	0
PRDX4	Peroxiredoxin-4	19.35	CDSN	Corneodesmosin	0
P4HB	Protein disulfide-isomerase	8.17	CAPN1	Calpain-1 catalytic subunit	0
HDAC1	Histone deacetylase 1	10.41	AGR2	Anterior gradient protein 2 homolog	0
MAPK1	Mitogen-activated protein kinase 1	4.53	RET	Proto-oncogene tyrosine-protein kinase receptor Ret	0
MCM5	DNA replication licensing factor MCM5	4.18	GHX9	ATP-dependent RNA helicase A	0
MCM3	Isoform 2 of DNA replication licensing factor MCM3	4.4	DHTKD1	Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1	0
MCM6	DNA replication licensing factor MCM6	5.35	RAB14	Ras-related protein Rab-14	0
HSD17B4	Isoform 2 of Peroxisomal multifunctional enzyme type 2	5.75	FUS	RNA-binding protein FUS	0
PHB	Prohibitin	4.37	GGCT	Gamma-glutamylcyclotransferase	0
LYZ	Lysozyme C	17.32	BLVRA	Biliverdin reductase A	0
ANXA1	Annexin A1	4.57	COL6A3	Collagen alpha-3(VI) chain	0
YWHAE	14-3-3 protein epsilon	4.53	GSN	Gelsolin	0
			LARS	Leucine--tRNA ligase	0
			HSD17B4	Peroxisomal multifunctional enzyme type 2	0
				Disintegrin and	
			ADAM33	metalloproteinase domain-containing protein 33	0
			DYNC1H1	dynein 1 heavy chain 1	0
			PGK1	Phosphoglycerate kinase 1	0
			PARG	Poly(ADP-ribose) glycohydrolase	0
			SLC9A3R2	Na(+)/H(+) exchange regulatory cofactor NHE-RF2	0
			TKT	Transketolase	0
			CKMT1A	Creatine kinase U-type	0
			NSF	Vesicle-fusing ATPase	0
			BTBD11	Ankyrin repeat and BTB/POZ domain-containing protein BTBD11	0
			AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1	0
			NARS	Asparagine--tRNA ligase	0
			RARS	Arginine--tRNA ligase	0
			RAB13	Ras-related protein Rab-13	0
			PDIA3	Protein disulfide-isomerase A3	0
			MVK	Mevalonate kinase	0

Red: Recapitulates findings reported in literature

Figure 61: Overlapping peptide numbers from affinity purification-mass spectroscopy experiments with pEGFP-C1, pEGFP-MIER3-C1, and pEGFP-MIER3-C1-LXXAA.

No evidence MIER3 interacts with a nuclear hormone receptor under conditions tested

To see if I could induce expression of nuclear hormone receptors and identify an interaction with MIER3, the previous experiment was repeated using serum starvation prior to transfection, followed by serum stimulation with 20% FBS following transfection. A list of additional peptide IDs identified by mass spectrometry unique to MIER3 and MIER3-LXXAA, but not present in the pulldowns using traditional media conditions, are listed in Table 28. A nuclear hormone receptor was not identified. However, this experiment recapitulated the previous results of HDAC1/2 and BAHD1 interactions, with the disappearance of BAHD1 in the LXXLL mutant pulldown.

MIER3 interacts with a wide array of signaling, transport, and metabolic proteins

The protein-protein interactions of MIER3, but not of the LXXLL mutant, were explored in a further affinity purification and mass spectroscopy experiment. The total elution from GFP empty vector and GFP-MIER3 pulldowns were run on an SDS-PAGE gel and bands individually excised and analyzed by LC-MS/MS (Figure 62). This resulted in detection of a greater number of peptide fragments per protein identifier and generated a more comprehensive list of interacting peptides. The greater number of peptide fragments that appeared with this method allowed for removal of IDs in which only one peptide was identified. Table 29 provides a list of these protein IDs following removal of peptides overlapping with the empty vector pull down and CRAPome-identified contaminants. SEQUEST software application assigned the tandem mass spectra to peptide sequences from databases of known protein sequences. From this, SEQUEST HT scores

Table 28. Additional mass spectrometry peptide IDs* that appear from serum-starved and stimulated GFP-Trap pulldowns unique to MIER3 and MIER3-LXXAA

MIER3			MIER3-LXXLL Mutant		
Gene	Description	Sequest HT	Gene	Description	Sequest HT
MIER3	Mesoderm induction early response protein 3	83.62	MIER3	Mesoderm induction early response protein 3	79.58
HDAC1	Histone deacetylase 1	21.81	HDAC1	Histone deacetylase 1	13.27
HDAC2	Histone deacetylase 2	17.5	HDAC2	Histone deacetylase 2	10.66
BAHD1	Bromo adjacent homology domain-containing 1 protein	1.68	HRNR	Hornerin	11.54
NOC4L	Nucleolar complex protein 4 homolog	5.81	NOC4L	Nucleolar complex protein 4 homolog	9.09
PFKM	Isoform 3 of ATP-dependent 6-phosphofructokinase	1.89	MESP1	Mesoderm posterior protein 1	0
HRNR	Hornerin	0	NEO1	Neogenin	0
ABCA9	ATP-binding cassette sub-family A member 9	0	NCBP3	Nuclear cap-binding protein subunit 3	0
LIPE	Hormone-sensitive lipase	0	PLSCR5	Phospholipid scramblase family member 5	0
SLC9A9	Sodium/hydrogen exchanger	0	FAM81B	Protein FAM81B	0
			SHROOM3	Protein Shroom3	0
			CAPN2	Calpain-2 catalytic subunit	0
			RIT2	GTP-binding protein Rit2	0
			HIVEP3	Transcription factor HIVEP3	0
			AMY1A	Alpha-amylase 1	0

*IDs not present in previous mass spectrometry of total elution or gel extraction

Red: Not unique to stimulation, but recapitulates previous findings

Figure 62: GFP and GFP-MIER3 band excision following affinity purification.

Coomassie blue staining of an SDS-PAGE gel run after a GFP-trap experiment using pEGFP-C1 and pEGFP-MIER3-C1. Wherever a band appeared in either the GFP-trap or GFP-MIER3-trap column, a corresponding region was excised in the other.

Table 29. Total peptide IDs unique to MIER3 from mass spectrometry of individual bands in GFP-trap pulldown SDS-PAGE gels

Gene	Description	# Peptides	Sequest HT
LMNA	Prelamin-A/C	27	140.31
MIER3	Mesoderm induction early response protein 3	15	536.21
ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	15	58.57
SLC25A13	Isoform 2 of Calcium-binding mitochondrial carrier protein Aralar2	14	59.72
HDAC2	Histone deacetylase 2	13	90.93
LMNB1	Lamin-B1	13	43.58
ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	13	39.78
HSPA1L	Heat shock 70 kDa protein 1-like	13	189.44
HDAC1	Histone deacetylase 1	13	90.36
ATAD3A	Isoform 2 of ATPase family AAA domain-containing protein 3A	12	40.16
STAT1	Signal transducer and activator of transcription 1-alpha/beta	12	44.25
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	11	29.99
SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	10	32.03
KPNA2	Importin subunit alpha-1	10	39.96
BAHD1	Bromo adjacent homology domain-containing 1 protein	10	75.8
PSMD2	26S proteasome non-ATPase regulatory subunit 2	9	39.52
MAGED2	Melanoma-associated antigen D2	9	38.65
ACTA1	Actin, aortic smooth muscle	9	65.3
AIFM1	Apoptosis-inducing factor 1, mitochondrial	9	21.26
OAS2	2'-5'-oligoadenylate synthase 2	9	32.12
TRIP13	Pachytene checkpoint protein 2 homolog	8	26.15
GALK1	Isoform 2 of Galactokinase	8	36.4
PRKDC	DNA-dependent protein kinase catalytic subunit	8	21.72
FAR1	Fatty acyl-CoA reductase 1	8	31.17
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	7	19.84
UNC45A	Protein unc-45 homolog A	7	16.56
SLC25A6	ADP/ATP translocase 3	7	43.51
HLA-B	HLA class I histocompatibility antigen, B-37 alpha chain	6	24.96
EHHADH	Peroxisomal bifunctional enzyme	6	25.37
FOXK1	Forkhead box protein K1	6	19.55
ARAF	Serine/threonine-protein kinase A-Raf	6	19.46
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	6	18.41
EHD4	EH domain-containing protein 4	6	16.52
ACSL3	Long-chain-fatty-acid--CoA ligase 3	6	13.31
FARSA	Phenylalanine--tRNA ligase alpha subunit	6	27.74
ESYT2	Isoform 2 of Extended synaptotagmin-2	6	19.58
MCCC2	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	6	16.94
SLC25A4	ADP/ATP translocase 1	6	40.82
CKAP4	Cytoskeleton-associated protein 4	6	16.16
ABCF3	ATP-binding cassette sub-family F member 3	6	19.81
FXR1	Fragile X mental retardation syndrome-related protein 1	6	25.21
IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	5	14.75
GFPT1	Glutamine--fructose-6-phosphate aminotransferase 1	5	13.75
NOP56	Nucleolar protein 56	5	13.39
SLC25A10	Isoform 2 of Mitochondrial dicarboxylate carrier	5	15.29
ACAD11	Acyl-CoA dehydrogenase family member 11	5	25.92
SUCLA2	Succinate--CoA ligase subunit beta, mitochondrial	5	13.15
ERLIN1	Erlin-1	5	16.72
DTX3L	E3 ubiquitin-protein ligase DTX3L	5	13.04

MARK2	Serine/threonine-protein kinase MARK2	5	16.2
LARP4B	La-related protein 4B	5	12.25
YME1L1	ATP-dependent zinc metalloprotease YME1L1	5	18.94
EHD1	EH domain-containing protein 1	5	11.54
FASN	Fatty acid synthase	5	12.63
SPTLC1	Serine palmitoyltransferase 1	4	9.67
IMMT	MICOS complex subunit MIC60	4	10.4
PARP9	Protein mono-ADP-ribosyltransferase PARP9	4	9.84
DNAJA1	DnaJ homolog subfamily A member 1	4	16.29
ZC3HAV1	Zinc finger CCCH-type antiviral protein 1	4	16.05
DYNC1L1	Cytoplasmic dynein 1 light intermediate chain 1	4	11.64
RAF1	Isoform 2 of RAF proto-oncogene serine/threonine-protein kinase	4	13.62
DHCR24	Delta(24)-sterol reductase	4	14.48
PSMC6	26S proteasome regulatory subunit 10B	4	9.94
MRPS27	Isoform 2 of 28S ribosomal protein S27, mitochondrial	4	9.03
RFC2	Replication factor C subunit 2	4	11.05
GNL3L	Guanine nucleotide-binding protein-like 3-like protein	4	10.22
SSH3	Protein phosphatase Slingshot homolog 3	4	8.34
LGALS3BP	Galectin-3-binding protein	4	14.86
CSDE1	Isoform 4 of Cold shock domain-containing protein E1	4	9.07
UGDH	UDP-glucose 6-dehydrogenase	4	10
SLC25A11	Mitochondrial 2-oxoglutarate/malate carrier protein	4	16.47
ZNF385A	Zinc finger protein 385A	4	9.2
TAP2	Antigen peptide transporter 2	4	9.59
KPNA1	Importin subunit alpha-5	4	17.33
CCNK	Isoform 4 of Cyclin-K	4	10.54
GTPBP4	Nucleolar GTP-binding protein 1	4	9.41
NDUFA10	NADH dehydrogenase 1 alpha subcomplex subunit 10, mitochondrial	4	12.12
SLC3A2	Isoform 4 of 4F2 cell-surface antigen heavy chain	4	12.83
CSE1L	Exportin-2	4	9.14
VPS51	Vacuolar protein sorting-associated protein 51 homolog	4	10.45
CDK1	Cyclin-dependent kinase 1	4	15.15
PDIA3	Protein disulfide-isomerase A3	3	6.6
PRPF6	Pre-mRNA-processing factor 6	3	7.54
RAB5C	Isoform 2 of Ras-related protein Rab-5C	3	7.99
AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1	3	9.76
COG4	Conserved oligomeric Golgi complex subunit 4	3	6.42
TPX2	Isoform 2 of Targeting protein for Xklp2	3	7.04
MCM4	DNA replication licensing factor MCM4	3	7.17
PURB	Transcriptional activator protein Pur-beta	3	7.27
WDR18	WD repeat-containing protein 18	3	6.39
ERAL1	GTPase Era, mitochondrial	3	7.56
IKBKB	Inhibitor of nuclear factor kappa-B kinase subunit beta	3	7.37
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	3	11.58
TXNDC5	Thioredoxin domain-containing protein 5	3	7.08
RBM39	RNA-binding protein 39	3	10.58
YWHAG	14-3-3 protein gamma	3	11.53
PES1	Pescadillo homolog	3	6.93
PDXDC1	Pyridoxal-dependent decarboxylase domain-containing protein 1	3	6.98
FKBP4	Peptidyl-prolyl cis-trans isomerase FKBP4	3	6.78
VPS53	Isoform 4 of Vacuolar protein sorting-associated protein 53 homolog	3	7.25
UFL1	E3 UFM1-protein ligase 1	3	6.28
SLFN5	Schlafen family member 5	3	7.69
RBM4	RNA-binding protein 4	3	10.15

MLH1	DNA mismatch repair protein Mlh1	3	6.33
PADI3	Protein-arginine deiminase type-3	3	7.21
DNAJC7	DnaJ homolog subfamily C member 7	3	7.3
CTNND1	Catenin delta-1	3	6.74
HLA-C	HLA class I histocompatibility antigen, Cw-17 alpha chain	3	12.92
PTPN1	Tyrosine-protein phosphatase non-receptor type 1	3	7.48
HGH1	Protein HGH1 homolog	3	6.29
EPM2AIP1	EPM2A-interacting protein 1	3	6.72
GTF3C5	Isoform 3 of General transcription factor 3C polypeptide 5	3	6.49
SFXN2	Sideroflexin-2	3	10.76
PGM3	Isoform 3 of Phosphoacetylglucosamine mutase	3	10.17
FASTKD5	FAST kinase domain-containing protein 5, mitochondrial	3	6.58
PSMA7	Proteasome subunit alpha type-7	3	6.52
MYEF2	Myelin expression factor 2	3	6.31
SPTLC2	Serine palmitoyltransferase 2	3	8.21
TRAF4	TNF receptor-associated factor 4	3	9.52
OAS3	2'-5'-oligoadenylate synthase 3	3	7.07
PRDX2	Peroxiredoxin-2	3	19.35
TBL2	Transducin beta-like protein 2	3	8.45
ZNF281	Zinc finger protein 281	3	8.71
PKP3	Isoform PKP3b of Plakophilin-3	3	11.36
GLT8D1	Glycosyltransferase 8 domain-containing protein 1	3	7.76
HLA-E	HLA class I histocompatibility antigen, alpha chain E	3	8.24
TARDBP	TAR DNA-binding protein 43	3	9.61
POLD3	DNA polymerase delta subunit 3	3	9.18
YY1	Transcriptional repressor protein YY1	3	6.45
LPCAT1	Lysophosphatidylcholine acyltransferase 1	3	11.81
DNAJA3	DnaJ homolog subfamily A member 3, mitochondrial	3	20.3
EBNA1BP2	Probable rRNA-processing protein EBP2	3	7.32
SLC25A1	Tricarboxylate transport protein, mitochondrial	3	14.04
RAB25	Ras-related protein Rab-25	2	6.89
MCRS1	Isoform 2 of Microspherule protein 1	2	5.87
DHRS13	Dehydrogenase/reductase SDR family member 13	2	5.53
FAM83H	Protein FAM83H	2	5.22
NTPCR	Cancer-related nucleoside-triphosphatase	2	5.07
DNAJA4	Isoform 2 of DnaJ homolog subfamily A member 4	2	4.56
DUSP23	Dual specificity protein phosphatase 23	2	4.78
STAT3	Signal transducer and activator of transcription 3	2	7.27
TOR1AIP1	Torsin-1A-interacting protein 1	2	4.76
FIP1L1	Pre-mRNA 3'-end-processing factor FIP1	2	5.13
RCN1	Reticulocalbin-1	2	5.42
ECT2	Isoform 3 of Protein ECT2	2	4.88
TMPO	Lamina-associated polypeptide 2, isoforms beta/gamma	2	4.45
LMF2	Lipase maturation factor 2	2	4.61
EXOC3	Exocyst complex component 3	2	4.85
RFC3	Replication factor C subunit 3	2	6.22
CTSD	Cathepsin D	2	8.64
STOML2	Stomatin-like protein 2, mitochondrial	2	6.17
HADHA	Trifunctional enzyme subunit alpha, mitochondrial	2	5.95
MRE11	Isoform 3 of Double-strand break repair protein MRE11	2	6.16
SFXN3	Sideroflexin-3	2	4.81
SNRPA1	U2 small nuclear ribonucleoprotein A'	2	4.6
SFXN1	Sideroflexin-1	2	5.84
ZNHIT2	Zinc finger HIT domain-containing protein 2	2	4.45
SLIRP	SRA stem-loop-interacting RNA-binding protein, mitochondrial	2	8.67
ACAD9	Acyl-CoA dehydrogenase family member 9, mitochondrial	2	5.21

EXOC2	Exocyst complex component 2	2	4.2
ATP2C1	Isoform 7 of Calcium-transporting ATPase type 2C member 1	2	5.39
KIFC1	Kinesin-like protein KIFC1	2	3.89
GATA3	Isoform 2 of Trans-acting T-cell-specific transcription factor GATA-3	2	5.06
RAB5B	Ras-related protein Rab-5B	2	5.64
RANBP9	Ran-binding protein 9	2	4.41
CNDP2	Cytosolic non-specific dipeptidase	2	10.04
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	2	5.7
TRIM26	Tripartite motif-containing protein 26	2	4.38
MYL6	Myosin light polypeptide 6	2	4.78
GANAB	Isoform 2 of Neutral alpha-glucosidase AB	2	4.86
YARS	Tyrosine--tRNA ligase, cytoplasmic	2	4.06
RIOX1	Ribosomal oxygenase 1	2	6.35
TMEM165	Transmembrane protein 165	2	7.87
TFRC	Transferrin receptor protein 1	2	4.48
ELAVL1	Isoform 2 of ELAV-like protein 1	2	5.98
DPM1	Dolichol-phosphate mannosyltransferase subunit 1	2	10.2
PRPSAP1	Isoform 2 of Phosphoribosyl pyrophosphate synthase-associated protein 1	2	5.99
SRSF6	Serine/arginine-rich splicing factor 6	2	4.77
IKBKKG	Isoform 2 of NF-kappa-B essential modulator	2	5.36
SQSTM1	Sequestosome-1	2	6.15
ITPK1	Inositol-tetrakisphosphate 1-kinase	2	5.4
ELAC2	Zinc phosphodiesterase ELAC protein 2	2	4.5
AKAP8L	A-kinase anchor protein 8-like	2	5.02
ADAM15	Disintegrin and metalloproteinase domain-containing protein 15	2	5.27
DNAJC10	DnaJ homolog subfamily C member 10	2	4.65
TBK1	Serine/threonine-protein kinase TBK1	2	6.2
SNX1	Isoform 3 of Sorting nexin-1	2	4.33
DHCR7	7-dehydrocholesterol reductase	2	5
TIMM50	Isoform 2 of Mitochondrial import inner membrane translocase subunit TIM50	2	4.47
PKP2	Plakophilin-2	2	5.88
RALY	RNA-binding protein Raly	2	4.19
NDUFS3	NADH dehydrogenase iron-sulfur protein 3, mitochondrial	2	5.83
SMARCB1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1	2	5.09
GART	Trifunctional purine biosynthetic protein adenosine-3	2	11.62
EMD	Emerin	2	4.17
SLFN11	Schlafen family member 11	2	4.62
GGCX	Vitamin K-dependent gamma-carboxylase	2	4.18
CDK9	Isoform 2 of Cyclin-dependent kinase 9	2	10.8
AP2M1	AP-2 complex subunit mu	2	4.09
SLC27A4	Long-chain fatty acid transport protein 4	2	4.08
HOOK2	Protein Hook homolog 2	2	5.64
FARSB	Phenylalanine--tRNA ligase beta subunit	2	4.33
TBC1D5	Isoform 2 of TBC1 domain family member 5	2	4.5
NCAPH2	Isoform 4 of Condensin-2 complex subunit H2	2	4.38
RBFA	Putative ribosome-binding factor A, mitochondrial	2	3.92
TRAFD1	TRAF-type zinc finger domain-containing protein 1	2	5.42
ZW10	Centromere/kinetochore protein zw10 homolog	2	4.72
RHOT2	Mitochondrial Rho GTPase 2	2	4.75
CCDC47	Coiled-coil domain-containing protein 47	2	4.93
NLRP2	NACHT, LRR and PYD domains-containing protein 2	2	4.73
NEU1	Sialidase-1	2	4.13

RCOR1	REST corepressor 1	2	4.47
SMARCD2	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 2	2	4.82
POLDIP3	Polymerase delta-interacting protein 3	2	4.53
DNAJB12	Isoform 2 of DnaJ homolog subfamily B member 12	2	4.55
TRIM28	Transcription intermediary factor 1-beta	2	3.96
PSMB6	Proteasome subunit beta type-6	2	5.03
DNAJB2	DnaJ homolog subfamily B member 2	2	4.73
REPIN1	Isoform 2 of Replication initiator 1	2	5.43
AKAP10	A-kinase anchor protein 10, mitochondrial	2	5.15
GCDH	Glutaryl-CoA dehydrogenase, mitochondrial	2	4.97
CDC73	Parafibromin	2	4.36
RPN2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	2	4.14
ABCD3	ATP-binding cassette sub-family D member 3	2	7.75
MSH2	DNA mismatch repair protein Msh2	2	5.11
TAP1	Antigen peptide transporter 1	2	6
EXOC4	Exocyst complex component 4	2	5.46
EXOC5	Exocyst complex component 5	2	4.77
SSBP1	Single-stranded DNA-binding protein, mitochondrial	2	4.51
BUB3	Mitotic checkpoint protein BUB3	2	4.54
PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	2	4.01
DECR2	Peroxisomal 2,4-dienoyl-CoA reductase	2	4.89
TELO2	Telomere length regulation protein TEL2 homolog	2	4.06
STAU1	Double-stranded RNA-binding protein Staufen homolog 1	2	6.16
OXSRI	Serine/threonine-protein kinase OSR1	2	4.07
RAB5A	Ras-related protein Rab-5A	2	5.09
STIP1	Isoform 2 of Stress-induced-phosphoprotein 1	2	4.33
UMPS	Uridine 5'-monophosphate synthase	2	4.33
PRPF4	U4/U6 small nuclear ribonucleoprotein Prp4	2	4.42
FKBP5	Peptidyl-prolyl cis-trans isomerase FKBP5	2	6.68
ESRP1	Epithelial splicing regulatory protein 1	2	9.57

were used to evaluate the strongest candidate peptides, stemming from a cross correlation of experimental and theoretical spectra. CRAPome was used to calculate stringent fold change calculations (FC-B) based on spectral peptide counts and negative controls. The top 25% of peptides within each stringency criteria were compared, this being number of peptide fragments detected, SEQUEST HT score, and FC-B score. Protein IDs belonging to at least 2 of 3 of these stringencies were accepted into a final list of “real” interactors (Table 30). This list again shows HDAC1/2 and BAHD1 as some of the strongest interactors. Broadly it includes proteins involved in signaling, apoptosis, antiviral response, chaperones, chromatin interaction, nuclear transport, transcription factors, mitochondrial transport and metabolism, steroid and fat transport and metabolism, translation, membrane dynamics, and cell proliferation.

The list of top protein interactors based on our stringency criteria were used for all subsequent analysis into the function of MIER3. The Gene Ontology Consortium was used to analyze this list based on common biological processes (Table 31) and shared molecular functions (Table 32). The independent ontologies provide a fold enrichment for each process or function based on the number of proteins from the input compared to what would be expected of all proteins. P values are derived from stringent Bonferroni corrections. In evaluating biological processes, the largest fold enrichment stems from C4-dicarboxylate transport, due to 4 members of the mitochondrial solute carrier family. Other processes with large fold enrichments but encompassing a larger number of proteins from the input list are ribose phosphate metabolism, purine ribonucleotide metabolism, and purine-containing compound metabolic process, which include proteins prevalent in acyl-CoA

Table 30. Top 25% of mass spectrometry peptide IDs meeting Contaminant Repository for Affinity Purification (CRAPome) stringency criteria unique to MIER3 from digestion of individual bands in GFP-trap pulldown SDS-PAGE gels

Gene	Description	Sequest HT	FC B	# Peptides
MIER3	Mesoderm induction early response protein 3	536.21		15
LMNA	Prelamin-A/C	140.31	26.86	27
HDAC2	Histone deacetylase 2	90.93	6.73	13
HDAC1	Histone deacetylase 1	90.36	6.73	13
BAHD1	Bromo adjacent homology domain-containing 1 protein	75.8	10.58	10
SLC25A13	Isoform 2 of Calcium-binding mitochondrial carrier protein Aralar2	59.72	14.41	14
ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	58.57	15.37	15
IDH2	Isocitrate dehydrogenase, mitochondrial	51.32	10.58	
STAT1	Signal transducer and activator of transcription 1- alpha/beta	44.25	12.49	12
LMNB1	Lamin-B1	43.58	13.45	13
SLC25A6	ADP/ATP translocase 3	43.51		7
SLC25A4	ADP/ATP translocase 1	40.82		6
ATAD3A	Isoform 2 of ATPase family AAA domain-containing protein 3A	40.16	6.25	12
KPNA2	Importin subunit alpha-1	39.96	10.58	10
ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	39.78	13.45	13
MAGED2	Melanoma-associated antigen D2	38.65	9.62	9
GALK1	Isoform 2 of Galactokinase	36.4	8.66	8
OAS2	2'-5'-oligoadenylate synthase 2	32.12	9.62	9
SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	32.03	10.58	10
FAR1	Fatty acyl-CoA reductase 1	31.17	8.66	8
BAG3	BAG family molecular chaperone regulator 3	30.2	6.75	
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	29.99	11.54	11
FARSA	Phenylalanine--tRNA ligase alpha subunit	27.74	6.75	6
TRIP13	Pachytene checkpoint protein 2 homolog	26.15	8.66	8
ACAD11	Acyl-CoA dehydrogenase family member 11	25.92	5.79	5
EHHADH	Peroxisomal bifunctional enzyme	25.37	6.75	6
FXR1	Fragile X mental retardation syndrome-related protein 1	25.21	6.75	6
HLA-B	HLA class I histocompatibility antigen, B-37 alpha chain	24.96	6.75	6
PRKDC	DNA-dependent protein kinase catalytic subunit	21.72	8.66	8
AIFM1	Apoptosis-inducing factor 1, mitochondrial	21.26	9.62	9
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	19.94	7.7	
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	19.84		7
ABCF3	ATP-binding cassette sub-family F member 3	19.81	6.75	6
ESYT2	Isoform 2 of Extended synaptotagmin-2	19.58	6.75	6
FOXK1	Forkhead box protein K1	19.55	6.75	6
ARAF	Serine/threonine-protein kinase A-Raf	19.46	6.75	6
YME1L1	ATP-dependent zinc metalloprotease YME1L1	18.94	5.79	5
IFIT3	Interferon-induced protein with tetratricopeptide repeats 1	18.41	6.75	6
KPNA1	Importin subunit alpha-5	17.33	4.83	4
MCCC2	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	16.94	6.75	6
ERLIN1	Erlin-1	16.72	5.79	5
UNC45A	Protein unc-45 homolog A	16.56	7.7	7
EHD4	EH domain-containing protein 4	16.52	6.75	6
SLC25A11	Mitochondrial 2-oxoglutarate/malate carrier protein	16.47	4.83	4
DNAJA1	DnaJ homolog subfamily A member 1	16.29	4.83	4

MARK2	Serine/threonine-protein kinase MARK2	16.2	5.79	5
CKAP4	Cytoskeleton-associated protein 4	16.16	6.75	6
ZC3HAV1	Zinc finger CCH-type antiviral protein 1	16.05	4.83	4
ESRP2	Epithelial splicing regulatory protein 2	15.43	5.79	
SLC25A10	Isoform 2 of Mitochondrial dicarboxylate carrier	15.29	5.79	5
HADHB	Trifunctional enzyme subunit beta, mitochondrial	15.19	5.79	
LGALS3BP	Galectin-3-binding protein	14.86		4
IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	14.75	5.79	5
DHCR24	Delta(24)-sterol reductase	14.48		4
GFPT1	Glutamine--fructose-6-phosphate aminotransferase 1	13.75	5.79	5
RAF1	Isoform 2 of RAF proto-oncogene serine/threonine-protein kinase	13.62	4.83	4
ACSL3	Long-chain-fatty-acid--CoA ligase 3	13.31	6.75	6
ACSL1	Long-chain-fatty-acid--CoA ligase 1	13.27	5.79	
SUCLA2	Succinate--CoA ligase subunit beta, mitochondrial	13.15	5.79	5
DTX3L	E3 ubiquitin-protein ligase DTX3L	13.04	5.79	5
FASN	Fatty acid synthase	12.63	5.79	5
LARP4B	La-related protein 4B	12.25	5.79	5
EHD1	EH domain-containing protein 1	11.54	5.79	5
SSH3	Protein phosphatase Slingshot homolog 3		4.83	4
RFC2	Replication factor C subunit 2		4.83	4
PARP9	Protein mono-ADP-ribosyltransferase PARP9		4.83	4
DYNC1LI1	Cytoplasmic dynein 1 light intermediate chain 1	11.64	4.83	4
GNL3L	Guanine nucleotide-binding protein-like 3-like protein		4.83	4
UGDH	UDP-glucose 6-dehydrogenase		4.83	4
SPTLC1	Serine palmitoyltransferase 2		4.83	4

Bold: Top 25% in all 3 selection criteria

Red: Recapitulates findings reported in literature

Table 31. Gene ontology based on biological process of proteins meeting top stringency criteria

GO Biological Process	# Proteins	# Expected	Fold Enrichment	P value
metabolic process	49	28.93	1.69	1.88E-02
cellular metabolic process	46	26.23	1.75	2.34E-02
primary metabolic process	45	25.55	1.76	2.46E-02
organic cyclic compound metabolic process	29	10.84	2.67	1.71E-03
cellular nitrogen compound metabolic process	28	11.46	2.44	2.02E-02
response to organic substance	27	10.22	2.64	7.28E-03
cellular aromatic compound metabolic process	27	10.05	2.69	5.20E-03
nucleobase-containing compound metabolic process	27	9.22	2.93	9.06E-04
heterocycle metabolic process	27	9.88	2.73	3.69E-03
small molecule metabolic process	26	5.87	4.43	3.01E-07
phosphorus metabolic process	24	7.41	3.24	1.01E-03
phosphate-containing compound metabolic process	23	7.32	3.14	3.52E-03
interspecies interaction between organisms	21	7.22	2.91	4.60E-02
organophosphate metabolic process	17	3.04	5.59	6.13E-05
organic acid metabolic process	16	3.42	4.68	2.07E-03
organic cyclic compound biosynthetic process	16	4.15	3.86	2.55E-02
oxoacid metabolic process	16	3.35	4.78	1.56E-03
oxidation-reduction process	16	3.25	4.92	1.06E-03
nucleobase-containing small molecule metabolic process	16	1.76	9.07	2.03E-07
carboxylic acid metabolic process	16	3.04	5.27	4.17E-04
aromatic compound biosynthetic process	15	3.68	4.07	2.89E-02
heterocycle biosynthetic process	15	3.64	4.12	2.55E-02
nucleoside phosphate metabolic process	14	1.49	9.38	2.78E-06
nucleotide metabolic process	14	1.46	9.6	2.08E-06
small molecule biosynthetic process	13	1.98	6.55	8.18E-04
purine nucleotide metabolic process	11	1.14	9.63	2.08E-04
purine-containing compound metabolic process	11	1.26	8.75	5.39E-04
ribonucleotide metabolic process	10	1.11	8.97	1.82E-03
purine ribonucleotide metabolic process	10	1.06	9.46	1.13E-03
ribose phosphate metabolic process	10	1.15	8.7	2.38E-03
defense response to virus	9	0.73	12.37	5.65E-04
response to virus	9	1.03	8.77	9.34E-03
type I interferon signaling pathway	5	0.23	21.63	4.41E-02
cellular response to type I interferon	5	0.23	21.63	4.41E-02
C4-dicarboxylate transport	4	0.08	51.17	1.82E-02

Table 32. Gene ontology based on molecular function of proteins meeting top stringency criteria

GO Molecular Function	# Proteins	# Expected	Fold Enrichment	P value
protein binding	65	48.8	1.33	1.45E-02
organic cyclic compound binding	51	20.45	2.49	1.87E-10
heterocyclic compound binding	50	20.15	2.48	6.27E-10
catalytic activity	40	19.6	2.04	1.04E-03
small molecule binding	35	8.73	4.01	9.47E-11
nucleoside phosphate binding	33	7.36	4.49	3.31E-11
nucleotide binding	33	7.35	4.49	3.27E-11
anion binding	31	9.77	3.17	2.45E-06
carbohydrate derivative binding	26	7.72	3.37	3.28E-05
purine nucleotide binding	25	6.55	3.82	5.64E-06
purine ribonucleoside triphosphate binding	24	6.26	3.83	1.25E-05
purine ribonucleotide binding	24	6.5	3.69	2.58E-05
ribonucleotide binding	24	6.56	3.66	3.07E-05
adenyl nucleotide binding	23	5.34	4.3	3.30E-06
adenyl ribonucleotide binding	22	5.3	4.15	1.66E-05
ATP binding	22	5.09	4.32	7.86E-06
RNA binding	21	5.72	3.67	3.27E-04
cadherin binding	10	1.1	9.11	4.99E-04
cell adhesion molecule binding	10	1.84	5.44	4.54E-02
oxidoreductase activity, acting on CH-OH group of donors	6	0.45	13.27	2.20E-02
adenine nucleotide transmembrane transporter activity	4	0.11	35.66	2.11E-02
C4-dicarboxylate transmembrane transporter activity	4	0.05	73.55	1.61E-03
purine nucleotide transmembrane transporter activity	4	0.12	34.61	2.35E-02
nucleotide transmembrane transporter activity	4	0.13	30.97	3.52E-02
ATP transmembrane transporter activity	4	0.1	42.03	1.16E-02
purine ribonucleotide transmembrane transporter activity	4	0.11	35.66	2.11E-02
dicarboxylic acid transmembrane transporter activity	4	0.13	30.18	3.87E-02

synthesis, mitochondrial metabolism, and fatty acid metabolism. Additionally, the viral response proteins rank highly. The largest fold enrichments in analyzing molecular function of our input proteins largely include transmembrane transporter activity, necessary for the previous mitochondrial and fatty acid metabolic proteins identified. Ribonucleotide and nucleotide binding functions do not have as large of fold enrichments but encompass a much greater proportion of the input proteins.

MIER3 interacts strongly with nuclear proteins involved in DNA metabolism and chromosome organization

Within my list of top interactors, 25/69 proteins are located at the mitochondrion while 28/69 serve a nuclear function. Nuclear localization of MIER3 has been established [256], making the appearance of a large number of cytosolic and mitochondrial proteins perplexing. MIER3 has protein domains specific for tight protein-protein interactions, so MIER3 overexpression may promote a large non-nuclear presence and facilitate protein interactions that may not otherwise occur. As a result, I chose to further narrow my top interactors list by solely focusing on nuclear proteins to parse out the most biologically relevant interactions and provide greater insight into the nuclear function of MIER3. The nuclear proteins from the list of top interactors (Table 30) were input into ToppGene annotations, which provided more detailed gene ontology lists than the Gene Ontology Consortium. Table 33 lists the shared biological processes and Table 34 gives the molecular functions. The greatest number of input proteins participate in regulation of DNA metabolism and chromosome organization. The major function of these proteins is in chromatin and transcription factor binding, deacetylase activity, and catalytic activity on

Table 33. Gene ontology based on biological process of nuclear proteins meeting top stringency criteria

GO Biological Process	Genes from Input*	Genes in Annotation	P value	Bonferroni
DNA metabolic process	13	1080	8.537E-10	1.49E-06
chromosome organization	12	1287	8.222E-08	1.44E-04
regulation of response to stress	11	1671	0.00001049	1.83E-02
regulation of DNA metabolic process	10	389	9.266E-11	1.62E-07
negative regulation of cellular component organization	8	819	0.00001445	2.52E-02
apoptotic signaling pathway	8	669	3.277E-06	5.73E-03
regulation of response to biotic stimulus	7	556	0.0000108	1.89E-02
protein localization to nucleus	6	300	0.00000369	6.45E-03
telomere maintenance	5	167	3.725E-06	6.51E-03
telomere organization	5	180	5.372E-06	9.39E-03
double-strand break repair	5	251	0.00002681	4.68E-02
regulation of defense response to virus	4	84	6.166E-06	1.08E-02
positive regulation of double-strand break repair via nonhomologous end joining	3	15	1.206E-06	2.11E-03

* 29 total genes were input

Table 34. Gene ontology based on molecular function of nuclear proteins meeting top stringency criteria

GO Molecular Function	Genes from Input*	Genes in Annotation	P value	Bonferroni
chromatin binding	7	629	3.21E-05	8.41E-03
transcription factor binding	7	750	9.77E-05	2.56E-02
DNA-binding transcription factor binding	6	396	2.32E-05	6.09E-03
catalytic activity, acting on DNA	6	364	1.44E-05	3.78E-03
repressing transcription factor binding	5	75	8.66E-08	2.27E-05
RNA polymerase II-specific DNA-binding	5	311	9.21E-05	2.41E-02
transcription factor binding	5	311	9.21E-05	2.41E-02
histone deacetylase binding	4	128	3.90E-05	1.02E-02
histone deacetylase activity	3	45	4.18E-05	1.10E-02
protein deacetylase activity	3	47	4.77E-05	1.25E-02
promoter-specific chromatin binding	3	54	7.24E-05	1.90E-02
nucleosomal DNA binding	3	55	7.65E-05	2.01E-02
NAD ⁺ ADP-ribosyltransferase activity	3	30	1.22E-05	3.18E-03
RNA polymerase II repressing transcription factor binding	3	35	1.95E-05	5.11E-03
transferase activity, transferring pentosyl groups	3	56	8.08E-05	2.12E-02
NAD-dependent histone deacetylase activity (H3-K14 specific)	2	11	1.20E-04	3.13E-02
histone deacetylase activity (H3-K14 specific)	2	11	1.20E-04	3.13E-02
STAT family protein binding	2	9	7.84E-05	2.05E-02
Krueppel-associated box domain binding	2	3	6.57E-06	1.72E-03

* 29 total genes were input

DNA. Relevant to the potential role of MIER3 in a chromatin repressive complex, there are 7 proteins that directly bind chromatin: protein mono-ADP-ribosyltransferase PARP9, E3 ubiquitin-protein ligase DTX3L, bromo adjacent homology domain-containing 1 (BAHD1), heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC), signal transducer and activator of transcription 1 (STAT1), and histone deacetylase 1/2 (HDAC1/2). There are 11 proteins that participate in chromosome organization: DTX3L, BAHD1, HNRNPC, HDAC1/2, DNA-dependent protein kinase catalytic subunit (PRKDC), guanine nucleotide-binding protein-like 3-like protein (GNL3L), replication factor C subunit 2 (RFC2), lamin A/C (LMNA), pachytene checkpoint protein 2 homolog (TRIP13), and apoptosis-inducing factor 1 (AIFM1). There are an additional 4 proteins that participate in repressing transcription factor binding: STAT1, HDAC1/2, and importin subunit alpha-1 (KPNA2).

MIER3 interacts with co-repressor proteins that potentially facilitate heterochromatin formation and transcriptional regulation as a multi-protein repressive complex

Of the nuclear proteins, BAHD1, HDAC1/2, LMNA/LMNB1, STAT1, and KPNA2 are the strongest interactors. The binding of nuclear import adaptor protein KPNA2, and also the weaker presence of KPNA1, indicates this is the mechanism of MIER3 nuclear transport via its nuclear localization symbol. The interaction of MIER3 with HDAC1/2 and BAHD1 has been established [264, 578], and provide evidence of MIER3 existing in a repressive complex. HDAC1/2 are critical catalytic components to many corepressor complexes through their histone deacetylase activity, including Sin3A,

NuRD, and CoREST complexes [579]. BAHD1 has been shown to recruit proteins involved in heterochromatin formation and gene silencing [580]. The novel interaction with the transcription factor STAT1 may indicate the gene regions a MIER3-containing repressive complex is recruited to and downstream pathways that are modulated in hormone-responsive breast cancer. Additionally, interaction with nuclear lamins may indicate a role in positioning this complex, structural organization of chromatin, and further promotion of heterochromatin formation [581]. This model is depicted in Figure 63, whereby MIER3 is imported into the nucleus via KPNA1/2, docked at the nuclear lamina, and positioned as an adaptor protein in binding STAT1, HDAC1/2 via the ELM2 domain, and BAHD1 via an LXXLL motif. The lamins-MIER3 scaffold position chromatin and HDAC1/2 in such a way that chromatin is deacetylated, and BAHD1 recognizes H3K27me3 marks and facilitates further chromatin condensation. STAT1 may direct this repressive complex to specific gene regions and promote transcriptional silencing.

The top MIER3-interacting nuclear proteins have associations with breast cancer

I input the nuclear proteins from the top MIER3 protein interactors list into the co-expression analysis function within Toppgene, and the top hits were from three breast cancer microarray expression studies. This is interesting since I purposefully selected the breast cancer cell line T47D for analysis due to the potential role of MIER3 in breast cancer development. Nuclear proteins coinciding with a breast cancer association are listed in Table 35 [444, 568, 569]. Within this list are STAT1, HDAC1/2, KPNA2, and LMNA. This supports a functional role of this potential repressive complex in gene silencing and heterochromatin formation not only in normal gene regulation but in breast cancer.

Figure 63: Model of the function of a novel HDAC1/2-containing MIER3 repressive complex in heterochromatin formation and gene repression in breast cancer. KPNA1/2 facilitates nuclear import of MIER3 where it is docked at the nuclear lamina. MIER3 serves an adaptor function in binding STAT1, HDAC1/2 via the ELM2 domain, and BAHD1 via an LXXLL motif. The lamins-MIER3 create a scaffold that positions chromatin and proteins for histone deacetylation by HDAC1/2 and recognition of H3K27me3 marks by BAHD1 that facilitates further chromatin condensation. STAT1 may direct this repressive complex to specific gene regions and promote transcriptional silencing. Created in BioRender (biorender.com).

Table 35. Proteins meeting top stringency criteria associated with breast cancer and located in the nucleus

Gene	Description	Bertucci et al. 2006*	Van't Veer et al. 2002**	Mutarelli et al. 2008***
IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	✓	✓	
LMNA	Prelamin-A/C	✓	✓	
MAGED2	Melanoma-associated antigen D2	✓	✓	
SSH3	Protein phosphatase Slingshot homolog 3	✓	✓	✓
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	✓	✓	
TRIP13	Pachytene checkpoint protein 2 homolog	✓	✓	✓
STAT1	Signal transducer and activator of transcription 1	✓	✓	
KPNA2	Importin subunit alpha-1	✓	✓	✓
PARP9	Protein mono-ADP-ribosyltransferase PARP9	✓		
DTX3L	E3 ubiquitin-protein ligase DTX3L	✓		
PRKDC	DNA-dependent protein kinase catalytic subunit	✓		✓
DNAJA1	DnaJ homolog subfamily A member 1	✓		✓
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	✓		
ZC3HAV1	Zinc finger CCCH-type antiviral protein 1	✓		
BAG3	BAG family molecular chaperone regulator 3		✓	
UGDH	UDP-glucose 6-dehydrogenase		✓	✓
HDAC2	Histone deacetylase 2		✓	
GNL3L	Guanine nucleotide-binding protein-like 3-like protein			✓
RFC2	Replication factor C subunit 2			✓
LMNB1	Lamin-B1			✓

Top hits were from breast cancer microarray expression data using co-expression analysis from Toppgene

* P value 1.038E-7, Bonferroni 6.056E-4

** P value 1.382E-6, Bonferroni 8.062E-3

*** P value 9.039E-6, Bonferroni 3.100E-2

Discussion:

Previous work in our lab and data in this dissertation has associated Mier3 with DMBA-induced rat mammary cancer susceptibility [256]. *MIER3* is also increased in female breast tumors [582] and associates with lower overall survival and reduced relapse-free survival [583]. Here, I identified MIER3-interacting proteins in T47D luminal B breast cancer cells, which highlights a potential functional role of MIER3 in a complex that may promote heterochromatin formation and gene repression in breast cancer.

Although I utilized multiple cell lines to account for transfection issues, I repeatedly found MIER3 protein overexpression was only evident in a low number of cells, even with proteasome inhibitor treatment. This may be due to a stoichiometric imbalance, where overexpression of one member of a protein complex disrupts the molecular ratio that is otherwise normally kept in balance and leads to improper pathway modulation, abnormal complex formation, and cellular defects [584, 585]. Since native MIER3 levels are low, it could be that overexpressed MIER3, not in complex with other proteins, leads to toxic effects and is targeted for rapid degradation. This could be particularly true for BAHD1 which also has intrinsically low protein levels, suggesting that MIER3-BAHD1 containing complexes must be tightly regulated and overexpression could be deleterious. The lack of MIER3 protein expression could also be due to resource overload, whereby overexpression requires too large of cellular resources for translation, folding, or localization [584]. MIER3 is likely imported into the nucleus via the karyopherin subunit beta 1 (KPNB1) pathway of nucleocytoplasmic transport based on strong interactions with adaptor proteins KPNA1/2. The strong demand for this pathway could exhaust its components, leading to unnatural accumulation in the cytoplasm. Build-up of cytosolic MIER3 could lead to

promiscuous protein-protein interactions that are otherwise not biologically relevant [584]. It is important to note that intrinsically disordered proteins (IDPs), proteins lacking stable structures, enable non-physiological interactions [586, 587] and MIER3 is >50% intrinsically disordered according to the Protein Disorder Prediction System (PrDOS) [588]. IDPs also tend to have low native expression levels [589], such as MIER3, of which overexpression and promiscuous interactions could modulate improper pathways or lead to degradation. This may explain why a major portion of MIER3 interacting proteins found following affinity purification and mass spectroscopy are involved in mitochondrial and fatty acid metabolism and transport. The structured regions of MIER3 – a SANT domain, ELM2 domain, and two LXXLL motifs – facilitate strong protein interactions. Compounded with the known promiscuous interactions of IDP regions, these pathways may be a result of forced interactions that could lead to MIER3 degradation. There would need to be additional experiments to determine if MIER3 does naturally exist in the cytoplasm and if these non-nuclear proteins are biologically relevant and indicate a cytosolic function of MIER3.

In addition to the overall goal of identifying the protein-protein interactions of MIER3 as an insight into its biological function, the specific hypothesis was that the LXXLL sequence motifs of MIER3 would interact with NRs. This hypothesis stemmed from the classical definition of the LXXLL motif as originally identified in coactivator proteins that bind directly to NRs in their ligand binding domains (LBD) and is referred to as the NR box [590]. In this mechanism, a ligand binds the LBD of a class II NR which releases a corepressor and allows the alpha-helical LXXLL motif of the coactivator to bind the NR and participate in transcriptional regulation of target genes [590, 591]. Corepressor

proteins have extended LXXLL motifs while coactivators harbor the LXXLL [592]. MIER3 lacks the corepressor motif but possesses two LXXLLs. However, since no NR was identified in my mass spectrometry analysis, even following serum starvation and re-stimulation to induce NR expression, the function of the LXXLL motifs of MIER3 likely does not involve the canonical NR binding and transcriptional repression. A noncanonical role of the LXXLL motif was later identified, which showed the motif participates in protein-protein interactions with other regulatory proteins, including transcription factors and cofactors [593]. Thus, the LXXLL sequence is a protein recognition motif involved in transcriptional regulation in both NR-mediated and independent pathways. In my mass spectrometry analysis of MIER3 interactions, I found an interaction with BAHD1 which is reproducibly lost with the LXXAA double mutant that interrupts binding specificity. The specificity of the LXXLL motif for certain binding partners is complex, arising from the spacing and number of motifs which varies greatly between regulatory proteins, as well as the composition of the residues adjacent to the central motif [593, 594]. Thus, the double LXXLL motifs of MIER3 may modulate interaction with auxiliary transcriptional regulators given alternative tissue types, developmental, or disease states. However, BAHD1 appears to be a core interactor with MIER3 given reported interactions in human embryonic kidney (HEK293) and human proximal tubule (HPT) cells [578]. The MIER3-BAHD1 interaction in breast cancer T47D cells in this study further displays BAHD1 appears to be a critical binding partner specific to the LXXLL motif and may possess a functional role in MIER3-associated susceptibility to breast cancer.

MIER3 is known to localize to the nucleus [256], so I chose to focus this study of MIER3-interacting proteins on those with nuclear functions, with BAHD1, HDAC1/2,

LMNA/LMNB1, STAT1, and KPNA2 appearing as the strongest interactors. BAHD1 and HDAC1/2 have previously been reported to interact with MIER3, as well as a BAHD1 interaction with HDAC1/2, in other non-diseased cell lines [266, 578]. These proteins collectively may exist as core components of a nuclear complex with other more transient protein interactions. The bromo adjacent homology (BAH) domain of BAHD1 is present in a number of nuclear proteins and has been demonstrated to facilitate chromatin processes such as protein–protein interactions, recognition of methylated histones, and nucleosome binding [595-597]. In addition to MIER3 and HDAC1/2, BAHD1 has been demonstrated to interact with KRAB-associated protein 1 (KAP1) and heterochromatin protein 1 (HP1 α , β , γ) which induce heterochromatin formation, the methyl-CpG-binding protein MBD1, and histone-lysine N-methyltransferases SETDB1 and SUV39-H1 [580, 598-600]. This supports a hypothesis that BAHD1 exists in a multiprotein repressive complex. HDAC1/2 are established core catalytic components of three co-repressor complexes that modulate transcription – NuRD (nucleosome remodeling deacetylase) [601, 602], Sin3A [603], and CoREST (co-repressor of RE1-silencing transcription factor) [604]. The components of these HDAC1/2-containing complexes possess DNA/chromatin recognition motifs and interact with transcription factors to target the HDACs to chromatin [559, 605]. They incorporate different protein subunits which gives underlying specificity for genomic loci with context-dependent biological functions [560]. BAHD1 and MIER3 share domains found in other proteins within HDAC1/2 co-repressor complexes. For example, the metastasis-associated protein (MTA) subunits [606, 607] and RERE/atrophin-2 [608, 609] possess BAH domains and juxtaposed ELM2 and SANT domains. Thus, MIER3 and BAHD1 could serve similar co-repressive functions by creating a core scaffold that recruit

HDACs via the established ELM2 domain [266], as well as combinatorial assembly of DNA binding transcription factors and chromatin modifying proteins to create a novel HDAC1/2 co-repressor complex.

Based on the established role of MIER1 in breast cancer development as part of a chromatin repressive complex, as well as the sequence homology to MIER3 and its association with breast cancer, MIER3 is projected to share a similar repressive role, though differing protein interactions may target a MIER3-complex to alternative loci and downstream pathways than MIER1. The function of HDAC1/2 and BAHD1 on histone modifications and recognition of repressive marks further indicates a MIER3-containing complex may have a role in facultative heterochromatin formation. This condensed higher order chromatin structure reduces access by transcriptional machinery and is achieved by the combination of functionally distinct proteins in complex that coordinate epigenetic modifications such as methylation of DNA and histone modifications [555, 558]. Maintenance of various regions of facultative heterochromatin is crucial between differing cell types, which give rise to alternative cellular functions. Therefore, deregulation of chromatin structure or the co-repressive proteins that promote it can have a role in various diseases, including breast cancer [610-612].

My analysis identified the nuclear lamins as strong interactors with MIER3 which further supports a chromatin co-repressor function of a MIER3-complex based on nuclear lamins' established role in chromosome positioning, epigenetic regulation of chromatin, and promotion of heterochromatin formation [613]. The nuclear lamins classically participate in the nucleoskeleton that supports the nuclear membrane, but also serve as docking sites for chromatin, transcription factors, and protein complexes involved in

chromatin regulation [581, 614, 615]. Lamins participate in epigenetic regulation of DNA by binding histones and directly to DNA at scaffold/matrix attachment regions (S/MARs) [616-618]. The function of lamins in heterochromatin formation is observed in cells with LMNA mutations that results in a loss of heterochromatin from the periphery of nuclei [581, 613]. These lamin-bound heterochromatin regions are referred to as lamin-associated domains (LADs), which are characterized by low gene density, minimal transcriptional activity, and repressive chromatin marks [619-621]. Hundreds of these LADs are further classified as facultative LADs, as they are cell type- and differentiation state-specific [619], generating an additional layer of context-dependent specificity of a bound co-repressor such as MIER3.

In addition to the nuclear lamins, BAHD1 also possesses an established role in facultative heterochromatin formation. In fact, BAHD1 overexpression induces large-scale chromatin condensation and DNA hypermethylation [580, 599]. Lamins and BAHD1 are both strong interactors with MIER3 and both may work in conjunction in heterochromatin formation as either a component of or in positioning a MIER3-containing complex. Of the BAHD1-induced heterochromatin domains, or BAHD1-associated differentially methylated domains (BADs), 60% overlap with LADs at the nuclear periphery [599]. It is also reported that the size distribution (0.1–10 Mb) and the median sequence length (0.5–0.8 Mb) of LADs are very similar to that of BADs (0.3–6.5 Mb and median 0.5 Mb) [599]. BAHD1 has a further role in heterochromatin formation as the BAH domain is a H3K27me3 reader, a mark of facultative heterochromatin, and discriminates against H3K9me3, a mark of constitutive heterochromatin [622, 623]. The BAD regions largely appear at satellites, interspersed repeats, and intergenic regions [599], though BAHD1 is

also capable of directly binding to gene promoter regions, possibly by recognition of H3K27me3 marks, and recruiting repressive factors, thereby coordinating heterochromatin formation and gene silencing [580]. BAHD1 is known to bind HP1, MBD1, and HDAC5 [580], none of which appear as a MIER3 interactor. Thus, BAHD1 may exist in alternative repressive complexes that are context dependent, as these experiments were performed in HEK293 cells. Additionally, the MIER3-containing co-repressive complex may be larger and possess more heterochromatin factors than anticipated. For example, I did not identify any DNA methylation proteins in the MIER3 pulldowns in T47D cells, although methylation is an important factor in the formation of heterochromatin. However, MBD1 interacts with BAHD1 so may be present within a MIER3-containing complex despite no physical interaction with MIER3. It will be important to further investigate components of this repressive complex based on cell type, development, or disease state in order to marry the DNA methylation patterns, histone modifications, and transcription factor recruitment that drives the specificity and functionality of this complex.

The other core MIER3 interactors, HDAC1/2, are well characterized and possess an established role in facultative heterochromatin formation. They share ~86% amino-acid sequence identity, often heterodimerize, and have high levels of functional redundancy [624-626]. HDAC1/2 do not have a DNA-binding domain, so exert their deacetylase function by interacting with transcription factors or as members of multi-component complexes, in which DNA-binding factors tether them to certain genomic loci [556, 561, 627]. MIER3 may be an important tethering mechanism for this potentially novel complex, as it appears to bind the nuclear scaffold and the ELM2 domain has been established as crucial for HDAC1/2 binding in the MIER family [266]. The activity of HDAC1/2 in

transcriptional silencing requires specific complex assembly, and the exact composition and recruitment proteins defines the repression of particular genomic regions and target genes [555, 628]. Through histone deacetylation, HDAC1/2 makes chromatin available for subsequent modifications by other complex components [628]. Within the MIER3 complex, deacetylation may allow BAHD1 and other histone-modifying factors associated with it to add additional repressive marks. In this way, layers of repression are created which all contribute to heterochromatin formation.

Of the MIER3-nuclear interactors, signal transducer and activator of transcription protein1 (STAT1) appears as the strongest transcription factor interactor. The STAT family of transcription factors are cytokine-activated proteins that have a role in the hematopoietic and immune systems [629]. In the canonical pathway of STAT1 function, STAT1 is activated by IFN type I (IFN- α/β) or IFN type II (IFN γ) binding at membrane receptors [630], driving both downstream gene activation and repression [630, 631]. The type I pathway results in STAT1 and STAT2 trimerization with p48 to form the ISGF3 complex, which translocates into the nucleus and binds to interferon-stimulated response elements (ISREs) in the promoters of IFN α/β -inducible genes [631]. The type II pathway results in STAT1 homodimerization that translocates into the nucleus and binds gamma activated sequence (GAS) in the promoters of IFN γ -responsive genes [632-634]. STAT1 also participates in constitutive activation of genes even without IFN activation [630]. This duality in activation and repression is likely due to cross talk between the numerous pathways STAT1 coordinates and the different interactions with transcriptional modulators [630, 635, 636].

The interaction of chromatin-remodeling complexes with transcription factors is thought to provide the biological specificity for these complexes by targeting them to certain genomic regions (39). Conversely, it is theorized that complexes are targeted to these regions by recognition of specific histone/DNA modifications, in which transcription factors bind after and exert their function [637]. Despite not understanding the order of appearance, transcription factors are crucial to co-repressor complexes in providing specificity of function that differs between cell types, as well as establishing and maintaining transcriptional repression [558, 560]. The interaction of STAT1 with MIER3 seems similar to a reported interaction of STAT1 with protein inhibitor of activated STAT family (PIASy), in which the LXXLL motif of PIASy was found to be critical for repression of STAT1-mediated gene activation, although this motif does not facilitate the interaction with STAT1 or affect its DNA binding ability [591]. The authors proposed that PIASy may serve as an adaptor protein with the LXXLL motif facilitating the assembly of other components of a co-repressor complex with a functional role in repression of STAT1-mediated gene activation [591]. MIER3 may participate with STAT1 in a similar manner, with a MIER3-repressive complex modulating STAT1 downstream pathways. However, the complexity of STAT1 signaling makes it difficult to hypothesize which genomic locations are bound and what are the possible downstream effects.

STAT1 and BAHD1 appear to function in a shared pathway, although no physical interaction between them has been demonstrated. BAHD1, along with HDAC1/2 and the co-repressor protein KAP1, are recruited at the promoters of certain interferon-stimulated genes (ISGs) and promote gene silencing [638-640]. The virulence factor LntA from *Listeria monocytogenes* infection displaces this BAHD1-HDAC1/2 complex from

ISG promoters, de-repressing transcription [640, 641]. In this context, a BAHD1-HDAC1/2 complex is repressing transcription of ISGs that are normally expressed via STAT1-mediated transcription. Even though this was demonstrated in the context of listeriosis infection, it adds credibility to the premise that a MIER3-BAHD1-HDAC1/2 complex is participating in repression of STAT1 pathways. HDAC1 has also been shown to physically interact with STAT1 and modulate IFN- α -induced transcription [638]. There is a general requirement for deacetylation in STAT1-dependent transcriptional regulation in response to IFN- α/β and IFN- γ [638]. Although there are many regulatory complexes that contain HDAC1, a requirement for HDAC activity in STAT1-mediated pathways adds to a further potential functional role of a MIER3-containing co-repressive complex.

Since my T47D cells were not stimulated with IFNs, the contribution of STAT1 within a MIER3-containing complex likely diverges from its canonical role in interferon responses. There has been an increasing number of studies indicating that there are alternative mechanisms of STAT1 function compared to this standard model of IFN-induced STAT1 activation, including a role of unphosphorylated STAT1 (uSTAT1). In resting cells not stimulated with cytokines, STAT1 has been shown to be constitutively nuclear, accounting for up to 40% of total STAT1 [642]. In the nucleus, uSTAT1 can form stable homodimers [643] and uSTAT1 monomers are also capable of binding DNA [644]. uSTAT1 can form complexes with other transcription factors and accessory transcriptional regulators on DNA target sites, with specificity generated by the composition of the uSTAT1-complex [644]. Thus, uSTAT1 acts as a constitutive transcription factor and generates gene expression patterns entirely distinct from activated pSTAT1-homodimers or -heterotrimers [644]. Additionally, nuclear-localized uSTATs can promote

heterochromatin formation, acting not only as transcriptional regulators at the expression level but also at the epigenetic level. This pathway was initially investigated in *drosophila*, in which the only encoded Stat protein, Stat92E, was found to bind Hp1 in its unphosphorylated form and localize to heterochromatin [645]. Stat activation lead to disruption of this complex and chromatin de-condensation, while increasing levels of uStat led to higher levels of heterochromatin [645]. This non-canonical function was found to be conserved in mammals and humans, in which uSTAT5A and uSTAT3 have been shown to colocalize with HP1 α , stabilize this interaction at heterochromatin, and promote heterochromatin formation [646, 647]. Although this non-canonical uSTAT function has not been investigated for uSTAT1, the association of STAT1 with BAHD1 and HDAC1/2, along with their established interaction with HP1 and role in heterochromatin formation, elevate this as a likely candidate function of STAT1 within a MIER3-repressive complex. Additionally, the established role of STAT1 as a constitutive transcription factor independent of IFN signaling indicates STAT1 is mediating non-canonical gene targeting and regulation within a MIER3 complex.

The ability of the identified MIER3-interacting proteins to modulate heterochromatin formation, and thus transcriptional repression, has potentially important ramifications on cancer biology, and our specific interest, the etiology of breast cancer. The epigenetic landscape found in breast cancer is largely characterized by hypomethylation of DNA accompanied by formation of repressive chromatin domains that leads to gene silencing [562]. These chromatin changes occur early in disease initiation and are thought to promote tumorigenesis by silencing tumor suppressor genes [562, 648]. Thus, chromatin repressive complexes serve a clear role in breast cancer development. I

present my strongest experimental protein interactors with rational nuclear functions in my final model of MIER3 within a novel HDAC1/2-chromatin repressive complex in breast cancer. KPNA1/2 serve as adaptor proteins in a KPNB1 manner of MIER3 nuclear import. MIER3 is tethered to the nuclear lamin, and itself serves as an adaptor protein in binding BAHD1 via the LXXLL motifs, HDAC1/2 via the ELM2 domain, and STAT1 through an undetermined region. The lamins aid in positioning chromatin and organizing DNA-protein interactions. The order of DNA recognition remains unclear. Unphosphorylated STAT1 may lead targeted recognition of specific gene regions and promote transcriptional silencing. Additionally, BAHD1 may recognize and bind H3K27me3 marks, positioning HDAC1/2 for deacetylase activity and promoting facultative heterochromatin formation. The activity of this MIER3 co-repressive complex is potentially enhanced in breast cancer, which could contribute to greater levels of facultative heterochromatin and silencing of tumor suppressor genes.

CHAPTER VIII

CONCLUDING REMARKS

Breast cancer is a complex disease modulated by interactions between genetic and environmental factors. The laboratory rat serves as a highly relevant model of human breast cancer for its use in studying the genetic components of breast cancer and evaluating molecular factors driving natural disease initiation and progression. There are eight identified quantitative trait loci in rats that associate with mammary cancer susceptibility (Mcs). This dissertation focuses on two of these genomic regions, *Mcs3* and *Mcs1b*, through genetic mapping and preliminary studies of *Mcs3* and mammary gland expression analysis and mechanistic insight into *Mcs1b*-nominated breast cancer gene *MIER family member 3* (*MIER3*).

The *Mcs3* locus was initially mapped to a 29.4 Mb region on rat chromosome one which was too large to perform functional studies. As a precursory study, I selected candidate genes within *Mcs3* that had known associations with human breast cancer and evaluated their expression in *Mcs3*-resistant and susceptible rat strains. I identified p21-activated kinase (Pak1) as upregulated at the mRNA and protein level in a subset of carcinogen-exposed rat mammary glands prior to the development of any histopathological disease morphologies. These matched levels observed in human breast carcinomas and demonstrated a novel role of PAK1 in disease initiation. Although there was no effect of *Mcs3* genotype on Pak1 expression (and the gene region was later excluded from the *Mcs3*

locus by further mapping and narrowing), I elevated the rat as a valuable model to study the role of PAK1 in early human breast disease development.

Simultaneous to this study, congenic rat lines harboring differing portions of the *Mcs3* locus were developed and evaluated for retained mammary cancer susceptibility or resistance phenotypes. From this, I concluded that *Mcs3* is located at a 7.2 Mb region on rat chromosome 1: 143700228-150929594, which reduced the size of the locus by 75%. I also discovered a novel pleiotropic effect of *Mcs3* which confers reduced body mass in addition to mammary cancer resistance in those rats positive for this genetic region. In females, reduced body mass is dependent on separation from males, indicating an influence of pheromones and an environmental interplay with this genetic locus. Human GWAS-identified variants that map to this region are also associated with body mass; however, none are associated with breast cancer. Further studies into this QTL are relevant. The shared genetic associations with body mass in the human and rat and the modulation of mammary cancer susceptibility in the rat, suggesting that there may also be unidentified breast cancer susceptibility genes in the orthologous human regions.

Another *Mcs* QTL, *Mcs1b*, has been narrowed to only 1.2 Mb and overlaps with human breast cancer risk allele *5q11.2*. Encompassed in this overlap of the rat and human are two genes, *Map3k1* and *Mier3*. *Map3k1* is an established breast cancer gene so I chose to further our understanding of *Mier3* in rat mammary cancer by evaluating its expression at sequential pathological stages along a DMBA-induced model of mammary cancer development. I selected RNAscope to analyze the cell-specific expression and spatio-temporal context of *Mier3* transcript abundance in preserved mammary tissue. Evaluating mammary glands for various disease lesions typically requires fixing whole mounted

mammary glands, defatting, staining for epithelial structures, and excising and separately processing disease morphologies. However, I was unsure if this pre-screening process would be deleterious to mRNA integrity and the downstream quantifiability of the RNAscope assay. I developed a combinatorial method to pre-screen mammary glands for disease lesions followed by RNAscope assays. I proved that extended time and solvent exposures during whole mounting did not disrupt RNA integrity as the expression quantification of three separate genes was not different between mammary glands or carcinomas that were either processed immediately or whole mounted first. This is an important finding because *in situ* expression analysis is much more information rich than within homogenized tissue, but it is often critical to first observe or select for epithelial changes during normal mammary gland development, function, or disease progression.

Using RNAscope, I found that the luminal epithelial cells expressed the greatest levels of *Mier3*. This is critical given our knowledge of mammary cancer biology since these are the most proliferative cell types vulnerable to chemical insult and the majority of invasive ductal carcinomas, which account for 80% of diagnosed female breast cancers worldwide, demonstrate a luminal phenotype [114, 117, 218, 478]. Additionally, I found exposure to DMBA induces >2x greater *Mier3* expression in otherwise normal-appearing mammary ducts, indicating a potential role of *Mier3* early in disease initiation. Overall, I found *Mier3* expression significantly increases with each histopathological stage of a precursor-carcinoma disease sequence. This is a key finding that highlights a potential role of *Mier3* not only in disease initiation but in tumorigenesis and warrants further study into the role of *MIER3* in breast cancer development. Since I identified significant changes in *Mier3* expression throughout disease progression, suggesting a functional role in tumor

progression, I also evaluated *Mier3* expression in the normal (non-chemically-exposed) rat mammary gland during time windows of susceptibility during development and reproduction. These are critical windows in which the same factors that modify the developmental or growth profile of the tissue can alter or be utilized in carcinogenesis susceptibility later in life. *Mier3* abundance was highest in mammary epithelial cells during embryonic, neonatal, and pubertal development. *Mier3* expression levels in embryonic mammary buds even surpassed that found in mammary tumors. Expression substantially dropped by adulthood and did not rise to the levels found in early development during the reproductive cycle. This study showed for the first time that in addition to a role in cancer, *Mier3* potentially has a temporal role during normal embryonic and post-natal development of the mammary gland. Understanding the function of *Mier3* during this pivotal period then could elucidate its underlying function in cancer.

My studies coupling affinity purification of GFP-tagged human MIER3 with mass spectroscopy indicates MIER3 likely functions within a novel HDAC1 and 2-containing chromatin co-repressor complex. I identified a previously unknown function of the LXXLL sequence motifs of MIER3 in binding the heterochromatin-promoting protein BAHD1. I also show for the first time an interaction of STAT1 with MIER3. I hypothesize that this multi-protein complex serves to facilitate heterochromatin formation and downstream gene repression. This is important considering I found the most enhanced *Mier3* expression in embryonic mammary buds and mammary tumors. Transcriptional repression is critical in embryonic development for maintaining the proper transcriptional programming that orchestrate sequential events necessary for differentiation, and dysregulation in cancer can lead to silencing of tumor suppressors. Within this repressive complex, MIER3 appears to

function as an adaptor/scaffold protein. MIER3 binds the nuclear lamin, which serves to dock protein complexes and chromatin, as well as facilitates binding of regulatory proteins HDAC1/2, BAHD1, and STAT1 through strong protein-protein interacting domains. Adaptor proteins can have prominent roles in disease development since small changes in their expression can have substantial effects on protein complex formation and the biological outcomes they mediate. This may explain the higher *Mier3* expression found in *Mcs1b*-mammary cancer susceptible lines as compared to resistant [248]. Since endogenous levels of *Mier3* are normally low, greater *Mier3* levels in the susceptible strain could promote more co-repressive complex formation that drastically modify heterochromatin levels and global gene expression, accounting for the observed differences in cancer phenotypes. Additionally, since these genotypic differences in *Mier3* expression have thus far only been observed in mammary tissue, the role of Mier3 in cancer development appears to function in a mammary gland autonomous manner. This merits further work to elucidate the function of MIER3 specifically in human breast cancer as well as in mammary gland development, where the high expression levels I found indicate modulation of Mier3-induced disease susceptibility may potentially occur during this time period.

The subunit composition of complexes that modulate chromatin structure and transcription varies between different cell types and developmental stages, which provides functional specificity. Since gene expression patterns during embryogenesis are entirely different from a developed adult or a disease state, the components of the MIER3 complex I identified in a breast cancer cell line may be drastically different from those that exist during fetal or pubescent development. For example, I identified the transcription factor

STAT1 as one of the strongest MIER3 interacting proteins in hormone responsive breast cancer, but none of the STAT family members appear to have a role in mammary gland development during embryogenesis or pubertal epithelial expansion [649, 650]. There may be other development-specific transcription factors that interact with MIER3 lead to targeting of alternate genomic sites and downstream pathways. Studies with the protein interactors of the MIER family and BAHD1 utilized numerous cell lines, which indicate HDAC1/2 and BAHD1 are core components of a MIER3 complex [266, 578]. The interaction of HDAC1/2 with lineage-specific transcription factors suggests a role in modulating different transcriptional programs, and indeed HDAC1/2 is downregulated in certain cells during differentiation when the repression of lineage-specific genes is no longer needed [627, 628]. The mammary gland does not become differentiated until the first reproductive cycle, so HDAC1/2, possibly within a MIER3-containing co-repressor complex, may be crucial in maintaining an undifferentiated epithelium during embryonic through pubertal development. The other core protein interactor of MIER3, BAHD1, has been demonstrated to modulate expression of estrogen receptor (*ESR1*) and progesterone receptor (*PGR*) in combination with MIER1/MIER3 [578]. Since estrogen and progesterone are critical in signaling morphological tissue changes and differentiation of the mammary epithelium, epigenetic silencing by way of a MIER3-BAHD1 complex may contribute to regulation of correct temporal development. Future work investigating the mechanism and function of MIER3 would need to evaluate the combinatorial assembly of MIER3-HDAC1/2-BAHD1 with different transcription factors and chromatin-regulatory proteins in mammary epithelial cells from embryonic, pubescent, and developed mammary glands.

I found the strongest MIER3-interacting proteins have a role in transcriptional repression via heterochromatin formation, dysregulation of which can have deleterious repercussions on breast cancer initiation and disease progression. Chromatin structure presents an additional layer of regulation of gene expression, but loss of epigenetic control by way of histone modifications or DNA methylation can lead to alterations in expression that result in cellular transformation and proliferation [562, 651]. Epigenetic deregulation is complex, as perturbations at the level of establishment, recognition, maintenance, and removal of chromatin marks can all contribute to oncogenesis [652]. The role of chromatin-repressive complexes in disease initiation can stem from either deregulation of protein subunits within the complex, which alters the balance of transcriptional activation and repression and promotes oncogenic transformation, or downstream epigenetic silencing of tumor suppressor genes, which is a central hallmark in the development of cancer [554, 556, 561]. The epigenetic landscape of breast cancer cells is comprised of global hypomethylation of DNA alongside repressive chromatin domains that leads to gene silencing [562]. These changes to chromatin structure occur early in breast cancer development and are thought to promote disease progression by silencing tumor suppressor genes [562, 648]. Thus, chromatin repressive complexes have a clear function in breast cancer development. The increase in *Mier3* expression I found throughout mammary cancer progression, coinciding with our mass spectroscopy analysis that indicates its presence in a chromatin co-repressor complex, strongly suggests dysregulation of MIER3 may contribute to the repressive chromatin domains characteristic in breast cancer.

Organismal development requires precise control of transcriptional programs in a spatially and temporally regulated manner, allowing for establishment and maintenance of

various cell identities despite a single underlying genome [556, 560]. This requires proper chromatin structure that is achieved by chromatin modifying enzymes [579, 653]. During the process of differentiation, chromatin architecture transitions towards a heterochromatin configuration, highlighting the instructional role of chromatin repressive complexes in orchestrating differentiation and preventing adverse transcriptional events [654-656]. The mammary gland is unique in that the majority of its development occurs postnatally. As a result, proper mammary gland transcriptional programming must be tightly regulated and maintained throughout the lifetime of the mammal. For example, luminal epithelial cells have critical repressive marks in the virgin gland, but these are decreased throughout pregnancy which allow these cells to differentiate into a milk-secreting population and create a lactation-competent mammary gland [653]. Changes in repressive histone modifications during the reproductive cycle may indicate a reduced role of Mier3, which coincides with the substantially lower *Mier3* levels I found in mammary epithelial cells during the reproductive cycle as compared to early development. I found the highest *Mier3* levels in embryonic through pubertal development. Alterations in chromatin remodeling and transcriptional programs during these early stages of mammary gland development can disrupt the function of progenitor cells that drive ductal branching and proliferation, which predispose the mammary gland to later disease susceptibility [657]. Thus, gaining insight into the function of MIER3 on chromatin structure and transcriptional regulation during mammary gland development will be critical to our understanding of its role in the etiology of breast cancer.

Separate from temporal chromatin changes, the mammary gland also possesses chromatin structural differences between cell populations. Stemming from their functional

differences, the mammary epithelial subpopulations possess chromatin states that are as different as those between mammary epithelial and stromal cells [653, 658]. Luminal progenitor cells, which give rise to luminal epithelial cells of ductal or alveolar fate, are the adult mammary cell type that most closely resembles the chromatin architecture of fetal mammary stem cells [659]. This link between adult luminal epithelial cells and embryonic mammary cells is noteworthy since I found luminal epithelial cells possess the highest abundance of *Mier3* transcripts and *Mier3* abundance was highest during embryonic development. Because this cell population gives rise to the majority of female breast cancers, it will be important to identify the role of a MIER3 co-repressor complex in this cell type to define how chromatin remodeling drives tumorigenesis.

In conclusion, the laboratory rat will continue to be an important model to investigate genetically determined mechanisms of mammary cancer susceptibility, especially in studying *Mcs3* and *Mcs1b* loci, which may translate directly to human breast cancer susceptibility. I propose a role of MIER3 within a novel HDAC1/2-containing co-repressor complex and report an association with mammary gland development and disease progression. Continued evaluation of its regulatory function throughout development, possibly by differing combinatorial assembly of protein subunits, will be necessary in understanding its role in a neoplastic state.

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APPENDIX

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For research article: Duderstadt EL, McQuaide SA, Sanders MA, Samuelson DJ.
Chemical carcinogen-induced rat mammary carcinogenesis is a potential model of p21-activated kinase positive female breast cancer. *Physiological Genomics* 2021.
PMID: 33346690. DOI: 10.1152/physiolgenomics.00112.2020

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For research article: Duderstadt EL, Sanders MA, Samuelson DJ. **A Method to Pre-Screen Rat Mammary Gland Whole-Mounts Prior To RNAscope.** *Journal of mammary gland biology and neoplasia* 2021. PMID: 33866475. DOI: 10.1007/s10911-021-09484-5

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CURRICULUM VITAE

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Education

2016 – present	University of Louisville PhD, Biochemistry and Molecular Genetics GPA: 3.9/4.0 Qualifying Exam Passed: 5/2018, PhD expected spring 2021	Louisville, KY
2016 – 2017	University of Louisville M.S., Biochemistry and Molecular Genetics	Louisville, KY
2012 – 2016	Wittenberg University B.S., <i>Suma cum laude</i> , Biochemistry and Molecular Biology GPA 3.9/4.0	Springfield, OH

Research Experience

2016 – present	University of Louisville Doctoral student; Dissertation Advisor: David J. Samuelson PhD Dissertation: <i>Genetic and Functional Studies of Rat Mammary Cancer Susceptibility</i> i. Physical mapping of a rat mammary carcinoma susceptibility locus to identify candidate breast cancer variants/genes ii. Exploration of candidate cancer gene, <i>Mier3</i> , expression during rat mammary cancer progression and windows of susceptibility iii. Mechanistic analysis of Mier3 protein domain interactions	Louisville, KY
2014 – 2016	Wittenberg University Undergraduate Researcher; Advisor: Peter E. Hanson, PhD Project: <i>Preliminary Studies of the Synthesis of Dibenzoxanthenes for Use in Photodynamic Chemotherapy</i>	Springfield, OH

- i. One pot synthesis of substituted and halogenated derivative dibenzoxanthenes
 - ii. Structural analysis of compounds by NMR
 - iii. Testing of potential cytotoxic effects by measuring singlet oxygen production and DPPH antioxidant assays
- 2013 – 2014 **Wittenberg University** Springfield, OH
 Undergraduate Researcher; Advisor: Kristen K. Cline, PhD
 Project: *Concentrations of Copper and Chromium Metals in Treated and Untreated Water Samples from Ohio Counties*
- i. Chelation ion chromatography of treated and source untreated water samples
 - ii. Atomic absorbance spectroscopy to determine copper and chromium concentrations with respect to EPA standards, and recommendations to Ohio counties

Teaching Experience

2017	Teaching Assistant, Advanced Biochemistry, University of Louisville
2014 – 2016	Teaching Assistant, Organic Chemistry, Wittenberg University
2013 – 2014	Teaching Assistant, General Chemistry, Wittenberg University

Publications

Emily L. Duderstadt, Sarah A. McQuaide, Mary Ann Sanders, and David J. Samuelson.
 Chemical Carcinogen Induced Rat Mammary Carcinogenesis is a Potential Model of p21
 activated kinase (PAK1) Positive Breast Cancer. *Physiological Genomics* 2020,
 PMID: 33346690

Selected for APSselect, a collection from the American Physiological Society that showcases
 some of the best recently published articles in physiological research

Emily L. Duderstadt, Mary Ann Sanders, David J. Samuelson.
 A method to pre-screen rat mammary gland whole-mounts prior to RNAscope.
Journal of Mammary Gland Biology and Neoplasia 2021

Selected Posters and Presentations

EL Duderstadt. Genetic and Functional Studies of Rat Mammary Cancer Susceptibility.
 Research Conference Seminars, Department of Biochemistry & Molecular Genetics,
 University of Louisville, August 26, 2019 and September 14, 2020

EL Duderstadt, MA Sanders, DJ Samuelson. Expression of Breast Cancer Candidate Gene
MIER3 in Rat Mammary Tissue During Development and Disease Progression. Research
 Louisville, Louisville KY, July 2019

EL Duderstadt, SA McQuiade, DJ Samuelson. Overexpression of p21-activated kinase
 (Pak1) in Rat Mammary Cancer Tumorigenesis. Research Louisville, Louisville KY, July
 2017

Grant Applications

NIH F31 Fellowship

Identifier: OGMB190586, Submitted: December 2018

Project title: *Functional analysis of mesoderm induction early response, family member 3*

Awards and Honors

2020	Doctoral Dissertation Completion Award, University of Louisville Graduate School
2014	Alpha Lambda Delta National Honor Society
2014	Omicron Delta Kappa National Honor Society
2014	Mortar Board National Honor Society
2012 – 2016	Provost Scholarship, Wittenberg University

Work Experience:

2014 – 2016	Chemical Stockroom Assistant, Wittenberg University, Springfield, OH
2014	Reading Assistant for AP Exams (College Board), Cincinnati OH

Service

2017 – 2019	Volunteer, Kentucky Science Museum, Brain Days Outreach, Louisville, KY
2015 – 2016	Volunteer, Boonshoft Museum of Discovery, Springfield, OH
2013 – 2016	Pianist and coordinator, Campus Services, Wittenberg University, Springfield, OH