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Congenic and functional analysis of rat mammary cancer susceptibility.

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CONGENIC AND FUNCTIONAL ANALYSIS OF RAT MAMMARY CANCER SUSCEPTIBILITY

By

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DEDICATION

I would like to dedicate my thesis to my parents. None of my accomplishments would have been possible had it not been for their love and sacrifices.

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ABSTRACT

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Breast cancer is a complex disease affected by genetic, epigenetic and environmental factors. The genetic architecture of breast cancer comprises of high to low penetrance alleles. Although low penetrance alleles associate with a small change in an individual's risk to breast cancer, the total number of variants present and the high population frequency attributes to a much greater population based impact compared to rare high penetrance alleles. Animal models have been used to study these low penetrance modifier alleles in breast cancer. Different rat strains vary in their susceptibility to 7,12- dimethybenzanthracene (DMBA) induced mammary carcinogenesis, with the Wistar- Furth (WF) rat being highly susceptible and the Copenhagen (Cop) strain being almost completely resistant to it. Linkage analysis performed to identify quantitative trait loci associated with DMBA induced mammary carcinoma susceptibility in the WF and Cop rats led to the identification of mammary carcinoma susceptibility loci 1-4 *(Mcs1-4*). This dissertation focuses on *Mcs3* and *Mcs1b*. The *Mcs3* locus was predicted across two peak marker on chromosome *1 D1Mit11* and *D1Wox6*. My work helped to physically confirm the *Mcs3* locus and map it to a 25.8 Mb region on rat chromosome *1*. The Mcs1b locus was mapped to a 1.8MB region on rat chromosome *2*. denDekker *et al* 2012, showed that the *Mcs1b* locus and identified *Mier3* as an *Mcs1b* candidate gene. My studies showed, the proportion of luminal mammary epithelial cells (MECs) was higher in *Mcs1b* resistant mammary epithelial cell enriched extracts (MEC extracts) compared to susceptible MEC extracts. This observation

suggests that luminal MECs are potentially the target cells of DMBA induced carcinogenesis and future mechanistic studies could be conducted in luminal MECs. Expression assays on MEC extracts suggest that *Mitogen-Activated Protein Kinase Kinase Kinase 1* (*Map3k1*), *GC-Rich Promoter Binding Protein 1* (*Gpbp1*) and *Mesoderm Induction Early Response 1, Family Member 3 alternative 5' start site variant (Mier3-alt5P)* are *Mcs1b* candidate genes.

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INTRODUCTION

Breast Cancer Statistics

According to cancer statistics for 2016, breast cancer was the most frequently diagnosed cancer among women, preceded only by skin cancer, accounting for 231,840 new cases or 29% of cancer diagnosed [1]. A woman's risk of being diagnosed with breast cancer has increased from 1 in 11 in the 1970s to 1 in 8 in 2015. There are many factors contributing to the increase in breast cancer risk, which include longer life expectancy, and earlier diagnosis due to prophylactic measure [2]. Current and past research has focused on the detection and treatment of this disease, leading to a 34% drop in death rates since the 1990s [2]. However, despite these achievements, breast cancer remains the second leading cause of cancer-related death among women, accounting for 15% of cancer-related death or 40,290 cases [3]. Thus, there is a need for further research to understand the mechanism, and to identify risk factors associated with the development of breast cancer.

Breast Cancer Risk Factors

Breast cancer is a complex disease controlled by environmental, genetic and epigenetic factors [2]. An individual's risk to breast cancer is determined by many factors, including gender, age, family history, reproductive history, diet, alcohol use, lifestyle and environmental exposure [2, 4]. Gender accounts for the largest portion ofrisk to developing breast cancer, with women being more susceptible to breast cancer than men. Female breast cancer accounts for 99% of breast cancer cases diagnosed and breast

cancer-related deaths [3]. The following text will discuss some factors contributing to breast cancer risk in more detail.

Age

It is very uncommon for a woman in her 20s to be diagnosed with breast cancer, but risk increases with age and the probability of a woman developing breast cancer in her 90s being 1 in 5 [2, 5]. The prevalence of breast cancer is less than 10 cases per 100,000 for women under the age of 25 years. This prevalence increases 100-fold by the age of 45, making it the leading cause of cancer-related death in women between the ages of 20 to 59 [3, 6]. Breast cancer etiology varies with age. Although young women are less likely to develop tumors, the tumors that they do develop tend to be more aggressive. Younger women develop more estrogen receptor (ER)-negative tumors compared to older women. This trend continues until about 60 years of age [7-9]. It was also found that, for women above the age of 60, luminal A is the most common breast cancer subtype, while in women below the age of 40, luminal A is the least common subtype [3, 10]. Thus, age is not only an important factor in the development of breast cancer, but also in the prognosis and treatment of disease.

Radiation

Radiation exposure is also a strong risk factor for breast cancer, since breast tissue is very sensitive to radiation-induced carcinogenesis [11]. The current understanding of the relationship between ionizing radiation exposure and breast cancer risk was obtained from epidemiological studies of patients exposed to radiation for medical treatment and women exposed due to proximity to atomic bomb explosions [11, 12]. Age at exposure is inversely related to risk of developing breast cancer. This is thought to stem from the

degree of differentiation of breast tissue at age of exposure. In support, women below the age of 20 are more susceptible than women above the age of 45 [12, 13]. It was also found that the risk of developing breast cancer is proportional to the dose of radiation exposure [12, 14]. However, studies have shown that low-dose radiation, delivered over a period of time, e.g. under medical setting, can also have a long lasting cumulative effect that may cause breast cancer [13, 14]. Medical use of radiation is recommended only when the benefits outweigh the risks of exposure [15].

Alcohol Use

A number of epidemiological studies have shown that alcohol consumption increases breast cancer risk [16-23]. Results of two meta-analyses suggest that an addition of 10 g of alcohol per day increases risk of breast cancer by 10% [24, 25]. It is worth noting that alcohol consumption is a risk factor associated with ER+ tumors [26, 27]. The mechanism behind alcohol consumption and breast cancer development is not clearly understood. One hypothesis proposed is that alcohol contribute to breast cancer development by modulating sex hormone levels in women [28]. Another hypothesis proposed is that acetaldehyde, a known product of alcohol metabolism, is actually the main contributor to alcohol-induced carcinogenesis [29, 30]. Alcohol is an organic lipid solvent. Another theory proposed is that it may increase cell permeability to carcinogens by modulating cell membrane properties. Alcohol has also been shown to impair folate metabolism, thus influencing DNA methylation. Folate plays an important role in DNA methylation [29]. Alcohol intake may also enhance reactive oxygen species (ROS) production, modulate carcinogen metabolism, and inhibit DNA repair [31]. In part

because of the risk between alcohol consumption and breast cancer development, women are recommended to drink alcohol in moderation ≤1 drink/day [32].

Smoking

Studies have shown smoking increases breast cancer risk by 10% [33, 34]. It was found that a woman has a higher risk if they started smoking many years prior to first pregnancy [34-37]. Results from the Canadian National Breast Screening Study (NBSS) indicate that breast cancer is associated with duration (40 years vs nonsmoker) of cigarette smoking, with a hazards ratio (HR) of 1.57 (95% CI = 1.29 – 1.92). The HR for the intensity (40 cigarettes per day vs nonsmoker) of cigarette smoking was 1.21 (95% CI = 1.04 – 1.40). Breast cancer was also found to be associated with cumulative exposure (40 pack years vs nonsmoker: HR = 1.09; 95% CI = 1.06 – 1.13) and latency (40 years since initiation vs nonsmoker: HR = 1.31; 95% CI = 1.10 – 1.53) of cigarette smoking [38]. Breast cancer is not historically considered a tobacco related cancer, but *in vivo* and *in vitro* studies have implicated cigarette smoke induces breast carcinogenesis [39]. Cigarette smoke has many well documented carcinogens, and mammary tissue is capable of taking up many of these carcinogens, including polycyclic aromatic hydrocarbons and N-nitrosamines [40]. Mammary epithelial cells (MECs) are capable of bio-activating these compounds into molecules that induce DNA-damage and -adduct formation [37, 41]. *N-acetyltransferase-2* (*NAT2*) is a polymorphic enzyme responsible for detoxifying aromatic and heterocyclic amines present in cigarette smoke. Women with a *NAT2* slow acetylator phenotype have increased breast cancer risk [42]. Slow acetylators increase the exposure time to carcinogens, thus increasing susceptibility. The formation of DNA adducts were more common in slow acetylators [43]. Thus, genetics has a role in

cigarette smoke induced mammary carcinogenesis. This observation further proves that, breast cancer is a complex disease and many factors contribute to an individual's risk of being diagnosed with it.

Environmental Pollutants

The two environmental pollutants most commonly associated with breast cancer are polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). PAHs are genotoxic oxidants formed as a result of incomplete combustion and are found in tobacco smoke, diet, and indoor and outdoor air pollution, [44]. PAHs have been linked to breast cancer through several epidemiological studies [45-48] and have been shown to cause mammary cancer in animal studies [39]. PAH DNA adducts can cause DNA damage over the course of a few months to a year, depending on cell turnover rate [45, 49]. In addition, PAHs are lipophilic and can be stored in adipose tissue for a long time [47]. PCBs are found in lubricants, liquid sealants, flame retardants and electrical insulators [50]. Despite curtailing the production and distribution of PCBs by the Toxic Substances Control Act of 1976, PCBs remain persistent in certain environments due to improper disposal, leakage from industrial products, and chemical spills [51]. PCBs are classified as a group 1 carcinogen by the International Agency for Research on Cancer. PCBs are also lipophilic and accumulate in adipose tissue. The dioxin-like (coplanar) PCBs activate the aryl-hydrocarbon receptor (AhR) signal transduction pathway resulting in altered gene expression promotion of tumorigenesis [52-55]. On the other hand, non-planar PCB congeners induce oxidative stress, DNA damage and apoptosis in mammalian cells [56-60].

Lifestyle

Many lifestyle choices can impact an individual's risk to breast cancer. Not having children or having the first child after the age of 30, not breastfeeding, long term use of contraceptive pills, and the use of hormone replacement therapy increase risk to breast cancer [61]. High physical activity has been shown to reduce breast cancer risk in women, with an average of 20 to 25% reduction in breast cancer risk in women with a higher level of physical activity compared to women with lower level of physical activity [62-64]. In addition to physical activity, certain diet has been associated with improved breast cancer risk and disease progression [65]. A review by Davies *et al.* suggested that a diet high in fiber and low in fat might be protective against breast cancer progression and recurrence [66]. Another study showed that eating a minimum of five fruit and vegetable servings daily, while maintaining a physically active lifestyle, improved the 10 year survival rate of breast cancer patients [67]. The American Cancer Society suggests that overweight and obese people reduce weight gain, adopt a physically active lifestyle, have a diet rich in vegetables and fiber-rich food, and reduce fat intake to reduce chances of developing breast cancer [68].

Estrogen

In 1991, Women's Health Initiative (WHI) research initiated a 15 year study on the beneficial effect of post-menopausal Hormone Replacement Therapy (HRT) on heart disease, cancer and bone fracture. This study had to be stopped prematurely in 2002 due to the increased incidence of breast cancer, stroke, and heart complications in the estrogen alone and the combination estrogen/progesterone treatment groups [69]. The US National Toxicology Program classified estrogens as carcinogens, the International

Agency for Research on Cancer (IARC) also classified both estrogens and estrogen/progesterone combined post-menopausal therapies as known carcinogens [70]. Epidemiological, animal, and *in vitro* studies indicate endogenous estrogens are involved in breast carcinogenesis [71]. Evidence towards estrogen's role in breast cancer started with the observation that a double oophorectomy significantly reduces breast cancer risk [72]. Breast cancer risk factors, such as early onset of menarche (<12 years), late onset of menopause (>55 years), and having no children or having them late in life, are indicative of a lifetime of mammary tissue exposure to sex hormones [71].

Many mechanistic studies have been conducted to understand the role of estrogen-associated signal pathways in carcinogenesis. Estrogen increases *c-Myc* and *cyclin D1* expression inducing cell proliferation; whereas, antiestrogen agents have a converse inhibitory effect [73]. Estrogen increases *Bcl-2* and *Bcl-*X_L expression in breast cancer cells and inhibits apoptosis [74]. In breast cancer cells, estrogen stimulates interleukin 8 and vascular endothelial growth factor secretion, both of which can potentially induce angiogenesis. As opposed to this, antiestrogen tamoxifen decreases tumor angiogenesis in ER+ breast cancer cells [75-77]. Research into the role of estrogen in breast tumor formation has led to the development of several antiestrogens as the primary breast cancer treatment given that two third of breast cancer is ER+. Surgical oophorectomy was the method chosen to treat women diagnosed with premenopausal breast cancer prior to the development of antiestrogens. The American Society Clinical Oncology no longer recommend oophorectomy for the treatment of breast cancer due to the success of antiestrogen drugs [78]. The first of these antiestrogen agents was tamoxifen, which was introduced in the 1970s. It is the preferred choice of treatment due

to its efficacy and low price. It is also used as adjuvant therapy to prevent breast cancer recurrence in both pre and postmenopausal women [79]. The introduction of tamoxifen was followed by the rapid development of selective estrogen modulators (SERMs) and aromatase inhibitors (AI). SERMS, a group of compounds which are structurally similar to estrogens, can bind to and modulate estrogen receptor (ER) function in different tissues [80]. In addition to tamoxifen, the FDA has approved two other SERMs, toremifene and raloxifene. Toremifene has antiestrogen activity in the mammary gland. It is used to treat postmenopausal women with metastatic breast cancer. Raloxifene is a second generation drug with a secondary use in osteoporosis prevention [81]. Fulvestrant is a third-line treatment for postmenopausal women that have ER+ tumors. It modulates ER by promoting ER turnover in cells [82, 83]. In addition to SERMs, another class of agents for the treatment for breast cancer was developed based on the observation that breast cancer tissue has higher levels of estrogen compared to non-cancerous cells. Aromatase catalyzes the final rate limiting step of estrogen synthesis. AIs (e.g. anastrazole, letrozole and exmestane) are used to treat breast cancer in postmenopausal women [84].

Epigenetics

Many studies have implicated epigenetic changes in breast cancer pathogenesis. DNA methylation and post translational histone modification are the main epigenetic modifications associated with breast cancer. Hypermethylation of promoter CpG islands is one method of gene inactivation which could occur in the initial stages of breast cancer development. In one study, 802 breast tumors were examined by the Cancer Genome Atlas Network. This work resulted in identifying many genes that are differentially methylated [85]. Many of these aberrantly methylated genes play critical roles in

apoptosis, cell cycle regulation, tumor suppression, angiogenesis, epithelial-mesenchymal transition (EMT) and metastasis [86, 87]. Studies have also shown how methylation changes can be an early sign of developing breast cancer and correlate with the development of breast cancer [88]. Methylation of genes like GHSR can be used to differentiate breast cancer from normal breast tissue [89]. Studies also suggest that higher level of methylation is indicative of more aggressive metastatic breast cancer [90]. Hyper methylation of pro-apoptotic gene (*HOXA5, TMS1*), cell cycle inhibitors (*p16, RASSF1A*), and DNA repair genes (*BRCA1*) have been identified in metastatic breast cancer cells [91]. Distinct patterns of posttranslational histone modification have been observed in breast cancer. *DNA methyltransferases (DNMTs)* and *histone desacetyltransferase (HDAC)* are highly expressed in the promoter of the ER gene, inactivating ER in ER negative breast cancer [92, 93]. Histone modification leads to the down regulation of E-cadherin which is necessary for the initiation of EMT [94].

Genetics

The genetic component of breast cancer development was first noted with the observation that there is a higher prevalence of breast cancer in monozygotic twins of patients compared to dizygotic twins or siblings of patient. The incidence is also high in 1st degree relatives of breast cancer patients. This risk is greater for individuals with more affected relatives and is also greater if these relatives were diagnosed at a younger age [95-98]. Approximately 5 to 10% of breast cancer cases are autosomal dominant, following a Mendelian pattern of inheritance. Another 15 to 20% of breast cancer cases are familial (women with first or second degree relatives that have the disease). Among hereditary breast cancer cases, 30% are attributed to germline mutations in high

penetrance alleles of *BRCA1* and *BRCA2* [3, 99]. Studies over the past two decades have shown that the genetic component of breast cancer susceptibility is complex and comprises of genetic variation at many different loci. These susceptibility alleles differ in their population based frequency and the relative risk that they confer. The genetic component of breast cancer susceptibility comprises of high penetrance alleles, moderate penetrance alleles and low penetrance alleles. As shown in figure 1, high penetrance alleles confer a greater than 50% of the lifetime risk of breast cancer, moderate penetrance alleles confer a lifetime risk of 20% or more; whereas, low penetrance alleles confers a lifetime risk of 10 to 20% [100].

High Penetrance Alleles

High penetrance alleles affect individuals, usually at a younger age and are present in some families with a breast cancer history. Such families were subjected to linkage analysis and positional cloning to identify high penetrance alleles in *BRCA1* and *BRCA2*, two tumor suppressors which are involved in DNA repair [101, 102]. These genes are relatively rare in a given population, they have a cumulative frequency of 1 in 800 for *BRCA1* mutations and 1 in 500 for *BRCA2* mutations and they confer an 85% risk by the age of 70 [103, 104]. Mutations in *BRCA1* and *BRCA2* are responsible for approximately 84% of families with more than 3 members diagnosed with breast cancer at younger than 60 years of age [103]. Other high penetrance alleles have been identified by studying syndromes with inherited cancers as a symptom. *TP53* mutations were identified in Li-Fraumeni Syndrome and *STK11/LKB1* mutations found in Peutz-Jegher Syndrome [105, 106].

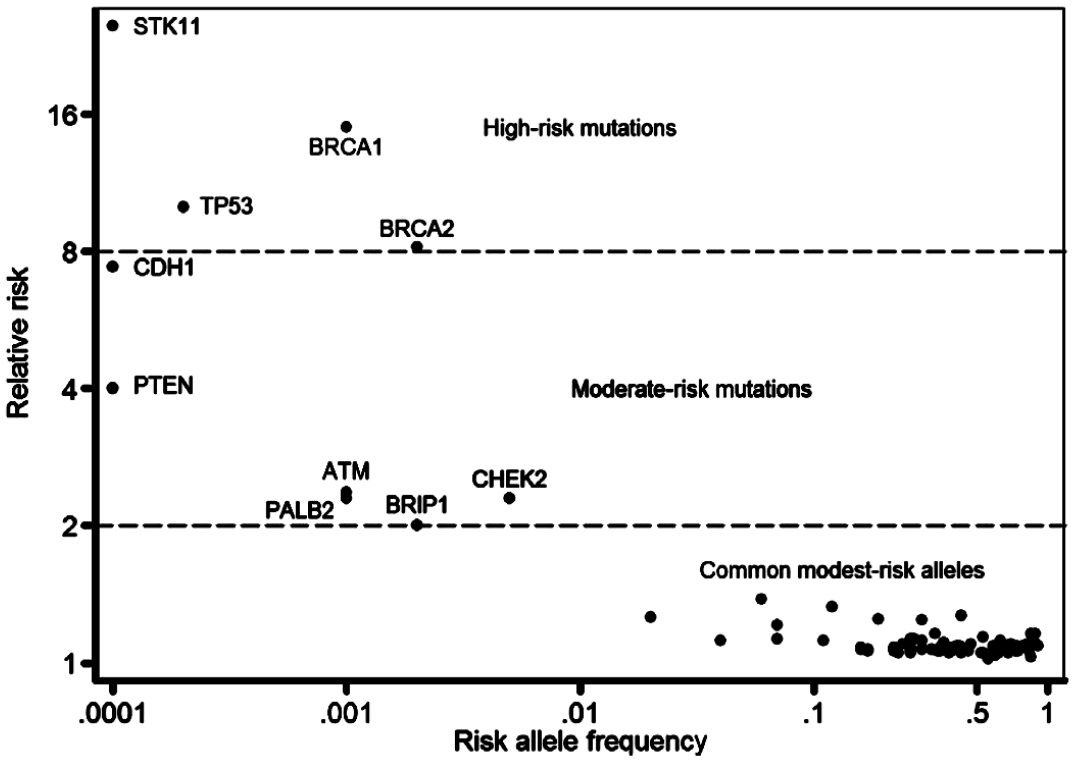


Figure 1. Genetic Architecture of Breast Cancer Susceptibility. Adapted from Ghouussani et al (2013). Figure shows the relative risk associated with different risk alleles as a plot against its respective allele frequency. Genes associated with breast cancer risk are further classified into groups based on level of risk.

BRCA1 and BRCA2

In 1990, *BRCA1* was the first major gene associated with hereditary breast cancer. It is present on chromosome 17 and was identified by linkage analysis of individuals in families with a history of breast cancer [107]. *BRCA1* is involved in homologous recombination to repair double stranded breaks in DNA [108]. *BRCA1* is also involved in cell cycle control, as an upregulation of *BRCA1* results in upregulation of p21 and G1-S cell cycle arrest [109]. In 1994, *BRCA2* was associated with increased risk to breast cancer in a genetic maping project focused on chromosome 13 [110]. Like *BRCA1*, *BRCA2* is also involved in DNA damage repair by homologous recombination [111]. Individuals with BRCA mutations are said to have Hereditary Breast/Ovarian Cancer Syndrome (HBOC). *BRCA1* related tumors tend to be ER, PR and Her2/neu negative while *BRCA2* related tumors are ER positive [112, 113]. BRCA mutations are inherited in an autosomal dominant fashion, but act recessively to bring about a phenotype of cancer. This is because loss of both copies is required, as these tumor suppressor genes are involved in double stranded DNA break repair [114]. *BRCA1* mutation carriers have a 10 to 40% lifetime risk whereas *BRCA2* carriers have 10 to 20% lifetime risk of ovarian cancer [115]. Inherited biallelic *BRCA2* mutations result in a Fanconi anemia type D1 like phenotype while inherited biallelic mutations in *BRCA1* are not documented, as this condition is likely embryonic lethal [114]. Women with BRCA mutations should be counseled regarding breast awareness and breast self-exam by 18 years of age. Annual MRI checkup is usually recommended from 25 years of age. Risk-reducing salpingo-oopherectomy (RRSO) is recommended after the age of 35 based on family history and

reproductive history. Prophylactic mastectomy is also frequently considered due to high lifetime risk and risk of secondary breast cancer [116-118].

Moderate Penetrance Alleles

Moderate penetrance alleles have been identified by resequencing genes that interact with *BRCA1* and *BRCA2,* or those which are a part of DNA repair pathways. Variants of *ATM, CHEK2, PALB2* and *BRIP1* show a 2 to 4 fold increase in breast cancer risk [119-123]. Linkage studies in families with rare syndromes that affect the breast, led to the identification of *PTEN* and *CDH1*. *PTEN* and *CDH1*. All confer a relative risk of two to eight [124, 125]. Altogether these genes account for less than 25% of risk associated with familial breast cancer [126].

CHEK2

CHEK2*1100delC is the most common CHEK2 polymorphism, present in 1 to 2% of European population. This gene can have a prevalence as high as 5% in breast cancer patients with a family history of breast cancer but *BRCA1* or *BRCA2* mutation negative [121] . CHEK2 is involved in cell cycle regulation at the G2 phase. It is a kinase that is phosphorylated in response to DNA damage. Activated CHEK2 interacts with BRCA2 while stabilizing BRCA1 [127]. Individuals who are co-carriers of *CHEK2* mutations and *BRCA1* or *BRCA2* mutations do not have an additional increase in risk as they induce overlapping effects on DNA repair [121]. As with *BRCA1* there is no known biallelic phenotype for *CHEK2*, indicating this condition may be embryonic lethal [114].

BRIP1

BRIP1 (BACH1) mutations account for less than 1% of breast cancer cases. Individuals with a history of breast cancer with *BRIP* mutations have a 2.0 relative risk of

developing breast cancer. These individuals have a higher risk for the early onset of breast cancer. BRIP1 interacts with BRCA1 C-Terminius (BRCT) domain of BRCA1. Most of the mutations identified are protein truncating. Individuals that are biallelic for *BRIP1* mutations are associated with Fanconi anemia type J; these patients do not have a significant increase in cancer during their childhood [119].

Low Penetrance Alleles

Low-penetrance breast cancer risk-associated alleles are fairly common in the population, many having a minor allele frequency of greater than 5% and each attributing to a small change in an individual's risk to breast cancer (relative risk of less than 1.5). Low penetrance alleles in the past have been identified in the past by case-control association studies, where candidate genes based on their known function are studied [128, 129]. This method is biased and may miss out on genes that are not yet functionally characterized. Since the advent of better genotyping technology, genome wide association studies (GWAS) have become possible. GWAS have led to the identification of over 70 breast cancer susceptibility loci. These loci however only explain 14% of familial breast cancer [100]. There is also some evidence that relative risk of low penetrance alleles are modified by environmental risk factors like life style and reproductive history [130]. Thus, there are many low penetrance alleles that remain to be identified.

Rs889312

5q11.2 SNP (*rs889312*) was one of the SNPS identified in a GWAS to associate with breast cancer susceptibility in a population with European ancestry [131]. The minor allele for *rs889312* was found to associate with a per allele odds ratio of 1.12 (95% CI =

1.10 – 1.15, $p_{trend} = 1.8x10^{-26}$) in the most recent Breast Cancer Association Consortium (BCAC) [132]. The association was strongest for ER+ disease (OR = 1.14, 95% CI = 1.11 – 1.17, $p = 1.1x10^{-26}$), but was also seen for ER- (OR = 1.06, 95% CI = 1.03 – 1.10, p = 0.0024) and triple negative disease (OR = 1.11, 95% CI = 1.02 – 1.20, p = 0.016) [133]. Carriers with *BRCA2* mutations associated with a greater risk of developing breast cancer if they carried the minor allele of SNP *rs889312* [134]. *Rs889312* is located in a disequilibrium block (LD block) which contains three genes, *MAP3K1*, *STED9* and *MIER3*. *MAP3K1* was considered as the candidate gene due to its driver mutations observed in Luminal A and Luminal B breast tumors and other breast tumor sub types [135, 136]. However, the study was not conclusive; and thus, any of the three genes or combination thereof within the LD block and any of the SNPs that are in linkage disequilibrium with *rs889312* could be the gene(s) or SNP(s) causing breast cancer susceptibility. Rats do not express a transcript of this gene [137]. *MIER3*, however, is more highly expressed in breast tumors compared to normal breast tissue, making it a candidate gene for a potential role in mammary carcinogenesis [137] This genomic region needs to be studied further to understand its role in female breast and rat mammary carcinogenesis.

Animal Models of Breast Cancer

There is no perfect experimental model to study breast cancer, but knowledge obtained from various models helps us to better understand this disease. Cell cultures are the simplest and most practical model to use. However, it is an oversimplified model and does not represent a complex *in vivo* tumor environment. Animal models come close in representing the multi-organ complexity of breast cancer. It is important to remember that

breast cancer represents an array of diseases and different experimental models are needed to represent and study different aspects of this array of diseases.

Breast Cancer Cell Lines

Breast cancer cell (BCC) lines used *in vitro* and *in vivo* studies have contributed significantly to the current knowledge regarding breast cancer. BCC lines, like any other model, have advantages and disadvantages. One of the main advantages is that it provides a homogenous, self-replicating model which can easily and quickly be cultured in simple growth media. BCC lines have been studied for more than 5 decades with the first cell line BT-20 described in 1958 [138]. Most BCC lines were established in the 1970s. A select few of them, namely, MCF-7, T-47D, MDA-MB-231 account for a majority of the research published [139]. Despite their role in understanding breast cancer, there are a lot of concerns regarding BCC lines being a representative model. It is usually the study of a single cell line which does not replicate or represent complex environments presented by the milieu of cells and interstitial fluid present in breast tissue or tumor environments. BCC lines are genetically unstable and long, stressful culture conditions can lead to its genotypic and phenotypic alteration. These changes lead to discrepancies in data interpretation and difficulties in replicating results [140]. One way of overcoming these short comings is by using an animal model to study breast cancer.

Canine Models

Mammary cancer is the second most common cancer in dogs. It is the most frequently diagnosed tumor in female dogs accounting for 52% of tumors diagnosed [140]. Canine mammary cancer is similar to human breast cancer with respect to tumor establishment and metastatic progression [141]. Canine mammary tumor like human

breast tumors are hormone dependent [142]. There is a significant overlap between canine and human genes that are deregulated in mammary cancer. Studies have shown that the deregulation of *BRCA1* and *BRCA2* are associated with mammary tumor development in dogs [143]. In another study, gene expression profiles from both human breast and dog mammary tumors were compared to normal breast and mammary tissue from the respective species. This study showed that in both species, similar pathways, related to increase in proliferation activity, were upregulated; whereas, similar pathways related to cell development, cell communication and cell matrix adhesion were down regulated. Cancer-related pathways like phosphatidylinositol 3-kinase (PI3K)/AKT pathway, KRAS, phosphatase and tensin homolog (PTEN), Wnt-b-catenin, and mitogen-activated protein kinase (MAPK) cascade were altered in both human and canine tumors [143]. The close similarities between canine and human mammary cancer makes dogs an appealing model to study human breast cancer. However, the disadvantages to using a canine model include its large body size, higher cost of housing, longer life span and fewer genetic tools available for manipulation as compared to its smaller, more easily housed rodent counterparts, which have a shorter life span.

Mouse Models

The mouse is an extremely popular model to study human breast cancer. The biology of mouse mammary gland has been studied and well characterized over many years with respect to normal development, changes occurring during lactation and developing spontaneous tumors. The popularity of the mouse as a model arises from its short lifespan, small size, feasible housing cost, ease of breeding in captivity and a well-defined genome [144].

Mice have been an extremely useful as xenograft and genetically engineered (GEM) models to study breast cancer. Several mammary specific gene promoters have been characterized enabling mammary specific engineering of genes. Several approaches have been used to genetically engineer mice [145]. One of the approaches is to create a transgenic (TG) mouse. Here, the gene of interest, like *C-myc* or *Ras* is randomly inserted into the germline by directly injecting DNA into one of the two pronuclei of a fertilized egg [146]. These mice have specific oncogene expression, usually controlled by mammary gland specific promoters. TG mice provide the option of testing human genes implicated in breast cancer in an *in vivo* setting [147]. Another mouse model which has been very beneficial to the study of breast cancer is the human to mouse xenograft model. Here, human cancer cells are grafted into the mouse resulting in a tumor with human cancer cells within the murine stromal cells [148]. Another approach, preferred to study tumor suppressor genes, like *BRCA1* and *BRCA2*, involves the generation of knock out mice. A nonfunctional version of the gene of interest is introduced into mouse embryonic stem (ES) cells. Cells with the targeted "knockout" gene, integrated by homologous recombination at the homologous site are selected. The resulting ES cells are used to generate the desired knock out mouse. A disadvantage of this model is that it is not an accurate representation of gene expression as most tumor-associated mutations are somatic and are only present in tumor cells and not globally expressed. Another disadvantage of this approach is that global inactivation of some genes often lead to embryonic lethality. This shortcoming led to the development of the next approach to be described where genes can be modified in the germline, but inactivated in a tissue specific and time specific manner. The most established method for tissue specific

knockout uses the Cre/Lox recombinase system [149]. LoxP are 34bp long genetic elements which are recognized by Cre recombinase enzyme. Knock in mice with two Lox sites on either end of the gene of interest are generated. These mice are still functionally normal despite the Lox elements. The knock in mice are then crossed with transgenic mice expressing Cre recombinase, which is controlled by tissue specific promoter, Cre mediated recombination occurs, resulting in tissue specific knock out of the gene of interest.

Despite its many advantages, a mouse model of breast cancer has its limitations. They do not best represent human tumors with respect to, growth over long periods of time, the role the immune system plays, and the heterogeneity observed in tumor cell, microenvironment and stroma [150, 151]. Another limitation observed is that mouse models are not predictive of tumor development in human tumors with the same histology [152]. Biologic differences, such as functionally active telomerase in murine cells unlike human cells, could affect oncogenesis [153, 154]. A majority of human breast tumors are estrogen responsive, while mouse mammary tumors are generally estrogen independent. Also, in most murine models, metastases occur hematogenously, and the primary site of metastasis is the lung. In humans, breast cancer is characterized by lymphatic and nodal metastases in addition to hematogenous metastases and sites of metastasis include lung, liver, bone, and brain [155].

Rat Models

Rats are another popular rodent model used to study breast cancer. Rat mammary cancer is very similar to human breast cancer with respect to histopathology, cell type of origin, and hormone responsiveness. The histologic features of malignant tumors in rats

are similar to intraductal and infiltrating ductal carcinomas in humans. Also, fibroadenomas (FA) and adenomas are similar in rats and humans [156, 157]. In rat mammary cancer, it was found that initiation of carcinogenesis occurs in the epithelium of terminal end buds (TEBs) while they are developing into alveolar buds (ABs) and terminal ducts (TDs). The TEBs in rat mammary glands are considered to be equivalent to the terminal ductal lobular unit (TDLU) in the human breast [156, 158]. Preexisting FA undergo rapid growth during pregnancy suggesting estrogen responsiveness. This pattern of rapid growth of ducts, cells lining ducts amount of periductal fibrous tissue found in FA is observed when both rat and human mammary glands are treated with high doses of estrogen[159].

Rat Models and Chemical Carcinogens

In addition to hormones, chemical carcinogens, like *7*,12-dimethylbenz[a]anthracene (*DMBA*) and N-Nitroso-N-methylurea (NMU), have been used experimentally to induce mammary cancer in rats. DMBA is usually administered as a single dose of 2.5 – 20 mg by oral gavage. DMBA needs to be bioactivated to induce carcinogenesis. NMU is also administered as a single dose of 25 – 50 mg/kg body weight by intravenous, intraperitoneal, or subcutaneous injections. Both carcinogens usually induce 100% tumorigenesis by 8-21 weeks [160]. Tumor number, latency and incidence are a function of carcinogen dose administered. [161]. DMBA or NMU induced carcinogenesis is age dependent and optimal at the age of sexual maturity which is 45 to 60 days of age in rats. Sexual maturity is marked by active organogenesis and glandular epithelial proliferation [162-165].

DMBA Induced Rat Mammary Carcinogenesis

In 1961, DMBA was reported to be a very specific and efficacious carcinogen to induce mammary carcinogenesis in female rats [166]. DMBA induced mammary carcinogenesis is dependent on the strain of the rat, age at DMBA administration, topographic location of the mammary gland and reproductive history [166, 167]. DMBA is a preferred carcinogen because it reliably induces mammary tumors and provides organ specificity; further, mammary tumors generated are of ductal origin and hormone responsive [168]. However, one limitation in the use of DMBA is that mammary carcinomas induced by DMBA do not metastasize [169]. DMBA rat model has provided several insights on the mode of carcinogenesis. DMBA induced carcinogenesis acts on the intermediate cells of the terminal end buds (TEB) resulting in intraductal proliferations and carcinoma *in situ* [156]. This provides an excellent model to study and identify mechanisms of breast cancer progression.

DMBA and Mammary Cancer Associated Quantitative Trait Loci (QTL) in the Rat Genome

Different strains of rats vary in their susceptibility to DMBA induced mammary cancer. This difference in susceptibility is not due to a difference in the ability of these strains to metabolize DMBA [170]. Studies have shown a genetic basis for the observed differential susceptibility to DMBA induced mammary carcinogenesis. The Wistar-Furth (WF) rat is highly susceptible while the Copenhagen (Cop) rat and Wistar-Kyoto (WKy) rats are highly resistant to DMBA induced mammary carcinogenesis [171]. Genetic linkage analysis performed on (WF × Cop) F1 × WF backcross (BC1) rats using tumor number post-DMBA administration as the phenotype. Microsatellite markers were used

to determine the genotype of rats that were phenotyped. This led to the identification of mammary carcinoma susceptibility locus 1 (Mcs1) on rat chromosome 2 as a locus that reduces mammary carcinoma susceptibility [172]. In an extension to this study, BC1 rats were further studied along with a linkage analysis performed on a panel of BC1 × BC1 backcross (BC2) rats and F2 (F1 × F1) rats to identify Mcs2, Mcs3, and Mcs4 on rat chromosome 7, 1 and 8 respectively. Mcs1, Mcs2, and Mcs3 loci reduce susceptibility of the susceptible strain, while Mcs4 increases DMBA induced mammary carcinoma susceptibility [173]. A similar linkage analysis, performed using the susceptible WF rat and resistant WKy rat, led to the identification of Mcs5, Mcs6, Mcs7, and Mcs8 on rat chromosome 5, 7, 10 and 14, respectively. Mcs5, Mcs6 and Mcs8 are associated with decreased susceptibility, while Mcs7 is associated with increased susceptibility to DMBA induced mammary carcinogenesis compared to the susceptible strain phenotype [174]. All loci except Mcs3 and Mcs7 have been studied beyond the initial mapping studies.

To study the Mcs loci congenic rats were generated using the scheme displayed in figure 2. These congenic rats contain only the Cop or WKy allele of interest on a completely WF genetic background. To generate congenic rats, homozygous rats of the parent strains are bred to generate heterozygous F_1 rats (Cop × WF or Wky × WF). The F1 rats are backcrossed with the susceptible WF parent to generate N2 rats. These rats are then successively backcrossed with the susceptible WF parent by selecting carrier rats with Cop or WKy allele of interest at each generation. Microsatellite markers and/or single nucleotide variants are used to genotype these rats to select carrier rats with the genotype of interest. The carriers are backcrossed for up to ten generations at which point the congenic rats usually only carry the Cop or WKy allele of interest on a completely

susceptible WF genetic background. These congenic rats are then used to study the Mcs locus of interest.

So far, *Mcs5* is one of the most studied Mcs locus. *Mcs5* was initially predicted as a locus that reduces mammary carcinoma susceptibility based on genetic linkage analysis

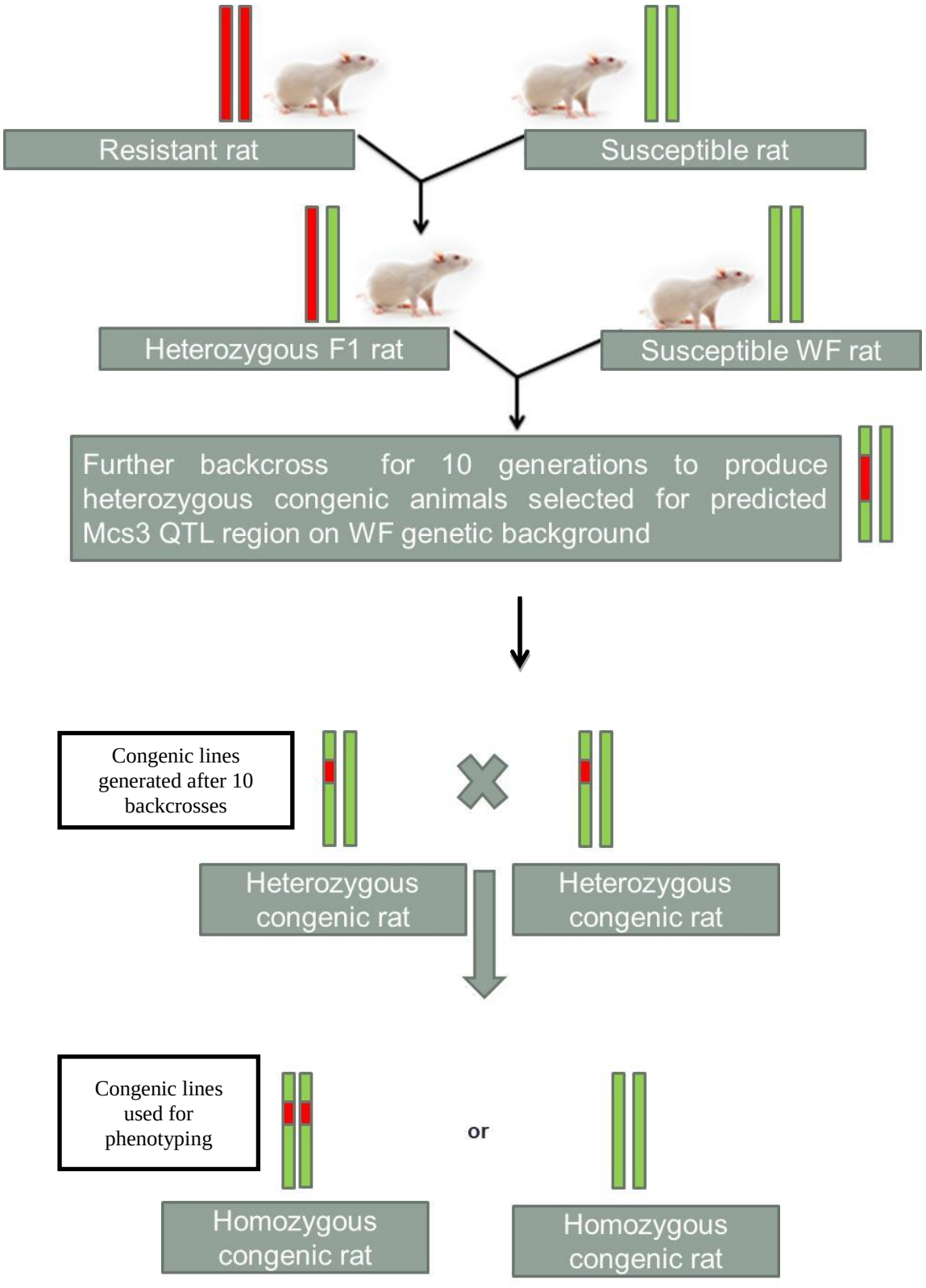

Resistant rat
Susceptible rat
Heterozygous F1 rat
Susceptible WF rat
Further backcross for 10 generations to produce heterozygous congenic animals selected for predicted Mcs3 QTL region on WF genetic background
Congenic lines generated after 10 backcrosses
Heterozygous congenic rat
Heterozygous congenic rat
Congenic lines used for phenotyping
or
Homozygous congenic rat
Homozygous congenic rat

Figure 2. Generation of Congenic Lines. Susceptible rat strain is crossed with a resistant rat strain. The F_1 generation is backcrossed with the susceptible parent strain, and subsequent offspring are backcrossed to the susceptible strain for ten generation by selecting for rats with the desired genotype. By the tenth backcross, the congenic rat with desired allele from the resistant rat strain, present on a completely susceptible genetic background is generated. The red and green bars represent the DNA from the different parent rat strains. The green bars indicate DNA from the susceptible rat strain and the red bars indicate DNA from the resistant rat strain.

performed on WKy and WF rats and was later physically confirmed as independently acting locus on rat chromosome 5 [174, 175]. Fine mapping this locus led to the identification of *Mcs5a*, *Mcs5b*, and *Mcs5c* loci. *Mcs5a* and *Mcs5c* reduce mammary carcinoma susceptibility while *Mcs5b* increases mammary carcinoma susceptibility in WF.WKy congenic rats compared to WF rats [176]. Studying each allele of these loci showed that neither the WF nor the WKy *Mcs5a* allele is dominant. *Mcs5a* and *Mcs5b* have an epistatic interaction where the *Mcs5a* allele masks the increased susceptibility of the *Mcs5b* allele. *Mcs5a* and *Mcs5c* interact synergistically to reduce mammary carcinoma susceptibility below the additive effect of the *Mcs5* alleles at each locus independently [176].

Fine mapping the *Mcs5a* locus showed that it contained 2 genetic elements, *Mcs5a1* and *Mcs5a2*. Thus, *Mcs5a* is a compound locus and is functional only when the Wky allele *Mcs5a1* and *Mcs5a2* are in cis confirmation on the same chromosome. *Mcs5a1* is located within *Fbxo10* and the *Mcs5a2* includes the 5' region of *Frmpd1*. *Fbxo10* was differentially expressed in thymus and *Frmpd1* was differentially expressed in the thymus of congenic rats compared to the WF control rats. Also, a study of the orthologous region in humans identified *MCS5A1* and *MCS5A2* as alleles with high population based risk and low population frequency [177]. *Mcs5a* Wky allele is associated with γδTCR+ T-cells proliferation and cytokine production in mammary epithelium exposed to carcinogens [178]. *Mcs5a* modulates mammary carcinoma susceptibility by a chromatin looping mechanism to down regulate *Fbxo10* expression in T cells in both humans and rats [179]. In a recent study it was found that *c-Fos* binds AP1- specific DNA elements in *Mcs5a1*, thereby regulating *FBXO10/Fbxo10* expression

by a PKC-dependent pathway [180]. *Mcs5a* is a prime example of how the rat has been used as a model to study the mechanism of human breast cancer susceptibility.

Mcs3

The work presented in this dissertation will focus on two different mammary carcinoma susceptibility loci, *Mcs3* and Mcs1b. In contrast to the *Mcs5* locus, which is well characterized, little is known about genetic variants associated with *Mcs3* and its underlying mechanism. *Mcs3* is a locus predicted on chromosome 1 across two peak markers *D1Mit11* and *D1Wox6* as shown in figure 3. *Mcs3* was predicted in a genetic linkage analysis using the WF and Cop rats. The *Mcs3* Cop allele was predicted to associate with reduced tumor number as compared to its WF allele [173]. Since, the *Mcs3* locus is predicted at two peak markers, which are 29Mb apart, its exact physical location and architecture may be complex. It may be a complex locus due to additive [181], dominant, epistatic, and synthetic [177] effects of low penetrance genes. The mechanism of action of a predicted locus and its complexity can be better identified by mapping the locus to as small a chromosomal region as possible. Also, for human studies it is convenient to map to a region of shared synteny. Thus, both physical confirmation and positional mapping of this region are necessary.

Mcs1b

The second part of this dissertation focuses on the *Mcs1b* locus. *Mcs1* rats developed an average of 1.2 tumors per rat post DMBA administration which was significantly lower than the WF rats which developed an average of 8.5 tumors per rat. *Mcs1* was confirmed as a locus that reduces mammary carcinoma susceptibility [172]. To analyze the complexity of the *Mcs1* locus, different recombinant congenic lines

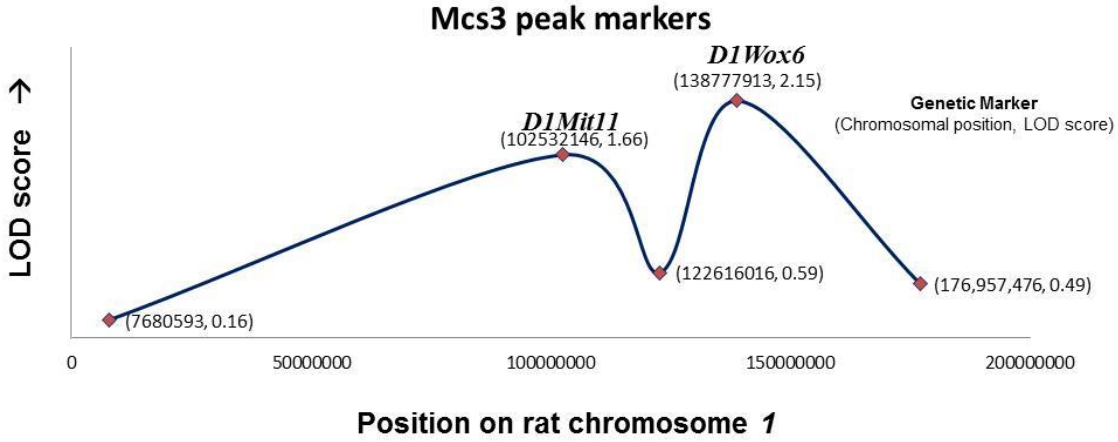


Figure 3. Representation of Predicted *Mcs3* Locus. Figure represents the Mcs3 locus which is predicted on rat Chromosome 1 across two peak markers *D1Mit11* and *D1Wox6*. The X-axis indicated the position on rat chromosome 1 and the Y-axis represents the LOD score.

with varying lengths of the Cop allele at the *Mcs1* locus were generated and phenotyped. This led to the identification of *Mcs1a, Mcs1b,* and *Mcs1c* loci, all of which reduce susceptibility to mammary carcinogenesis. [181]. Mammary gland grafting assays later showed that the *Mcs1b* locus acts in a mammary gland autonomous manner. The *Mcs1b* locus is of particular interest since it is orthologous to a GWAS identified locus associated with increased breast cancer incidence [131]. Haag *et al*. initially defined the *Mcs1b* locus between markers *D2Uwm17* proximally and *D2Rat16* distally (~13cM/11Mb) [181]. Recent work in our lab has delineated the *Mcs1b* locus to a 1.8 Mb region of rat chromosome *2* which is represented by congenic line N3 in figure 4 [137]. *Mcs1b* heterozygous congenic rats tested by Haag *et al*. developed an average of 7.6 tumors/rat [181]; whereas *Mcs1b* heterozygous congenic rats tested by denDekker *et al*. developed around 5.5 tumors/rat [137]. This discrepancy in tumors developed by rats that are heterozygous Cop at the 1b-11 locus observed in two different studies could be indicative of a complex genetic interaction within the Mcs1b locus. To continue fine-mapping *Mcs1b*, it is essential to generate recombinant congenic rats with smaller regions of the *Mcs1b* locus. Backcrossing line T (figure 4) generated the congenic line 1b-11 which spans the distal end of the *Mcs1b* locus. A preliminary number of 1b-11 rats have been phenotyped, but more 1b-11 (figure 4) rats need to be phenotyped to determine the effect of Cop 1b-11 allele on susceptibility to DMBA-induced mammary carcinogenesis.

Results from the same study by denDekker *et al.* that reported *Mcs1b* acts in a mammary gland cell autonomous manner, suggest that *Mcs1b* is a non-protein coding genetic element [137]. *Mcs1b* associated phenotypes include altered expression levels of *Map3k1, Il6st, Gpbp1* and *mesoderm induction early response1, family member3 (Mier3)*

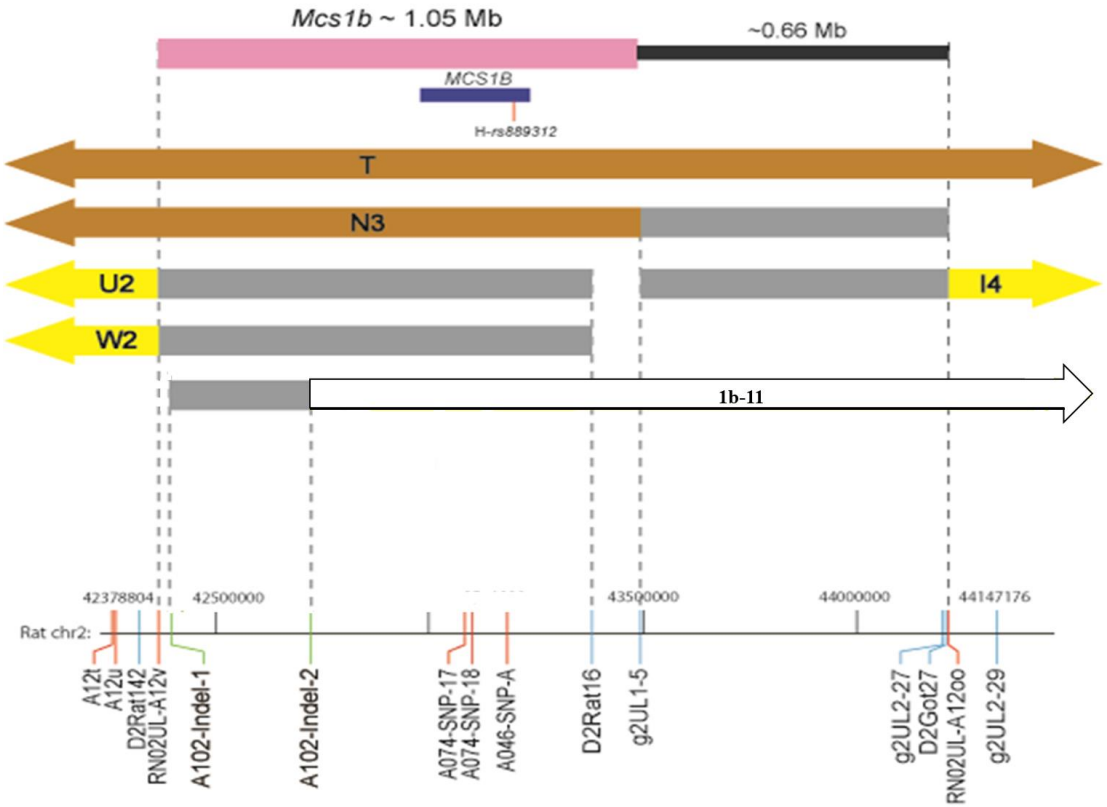


Figure 4. WF.Cop Congenic Map that Defines the *Mcs1b* Locus. Figure represents varying segments on rat chromosome *2* where *Mcs1b* was predicted to be located and congenic lines that were used to fine-map this locus. On the X axis, blue lines represent informative microsatellite markers, red lines represent informative SNPs, and green lines represent informative indels. Brown bars represent Cop alleles in congenic lines with a resistant phenotype, yellow bars represent congenic lines with a susceptible phenotype, white bars represent congenic lines with an unknown phenotype and grey bars represent a potential area of recombination where the genotype isn't determined. The horizontal blue bar represents the orthologous region to human *5q11.2* breast cancer risk (BRCR) associated haplotype block.

in mammary glands. Of all the genes contained within the *Mcs1b* region only *Mier3* was differentially expressed between the mammary glands of *Mcs1b* resistant and susceptible WF rats with or without DMBA administration. *Mier3* expression was 4.5 fold lower in mammary glands of the resistant congenic rats compared to susceptible WF rats [137]. These differential expression patterns were not observed in corresponding spleen, thymus, ovary or brain tissues; therefore, *Mier3* transcript levels vary specifically between susceptibility genotypes in mammary gland tissue. It was also found that *Mier3* transcript level was 1.8 times higher in mammary carcinomas as compared to surrounding disease-free mammary tissue; human *MIER3* levels were respectively 1.33 and 1.20 times higher in invasive ductal and invasive lobular carcinoma samples compared to normal breast tissue [137, 182]. Thus, *MIER3* is a potential candidate breast cancer susceptibility gene. *MIER3* is an uncharacterized gene with a protein product localized to the nucleus [137]. Immunohistochemistry staining for *Mier3* in mammary gland tissue of congenic rats showed that it is not uniformly expressed in all cell types but is specific to a sub-population of epithelial cells lining the duct (figure 5). It was also identified that an alternative 5' transcriptional start site variant of *Mier3* is differentially expressed between *Mcs1b* congenic and WF rat mammary glands compared to other *Mier3* variants [137]. Differentially expressed 5' start variants are typically indicative of cell or tissue type specific expression. Hence, it is possible that *Mier3* is either responsible for reduced susceptibility to mammary carcinomas conferred by *Mcs1b*, or it is a marker of a specific cell type in the mammary gland that is responsible for this phenotype.

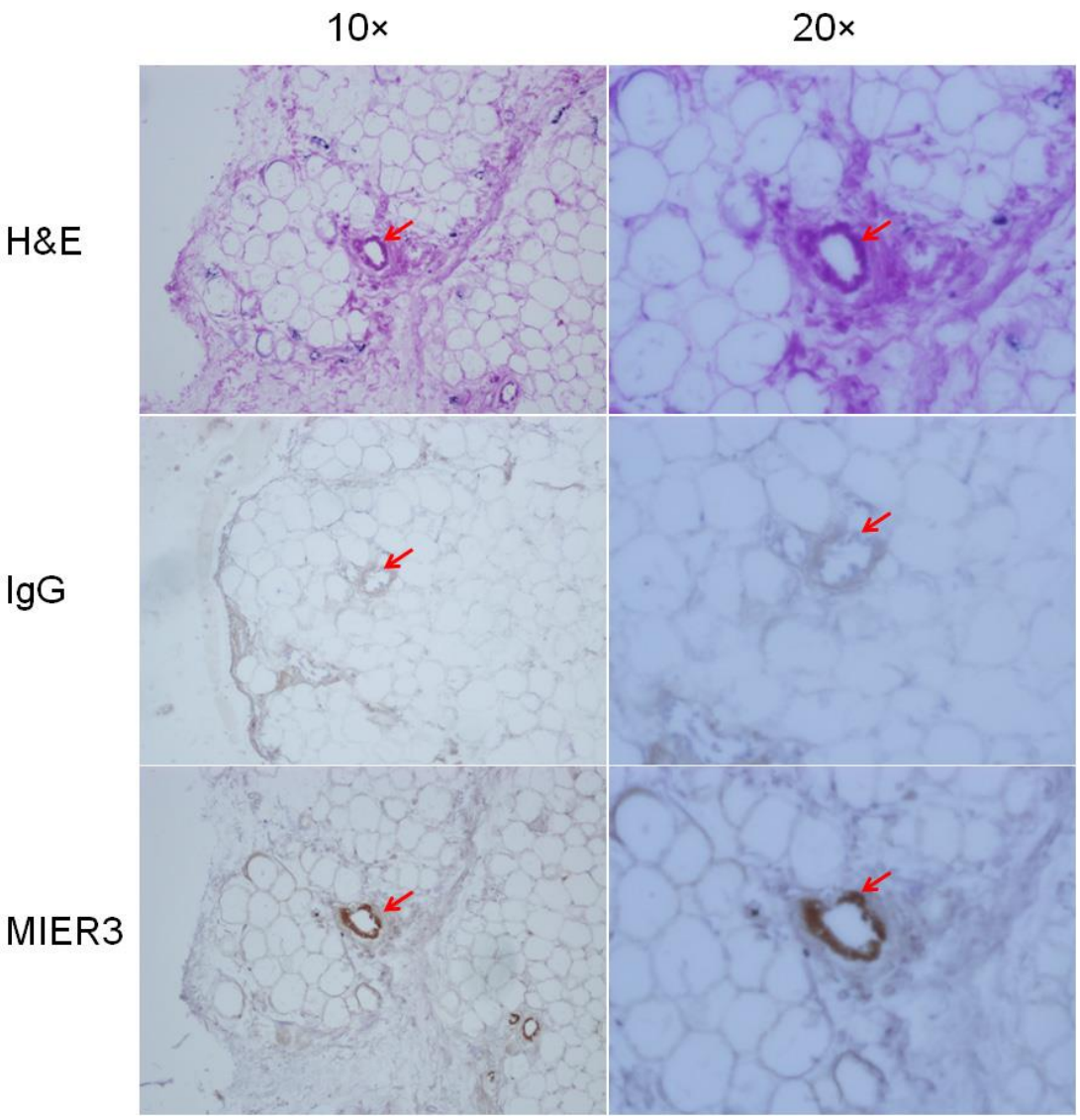


Figure 5. Expression of Rat *Mier3* in Cells Lining Mammary Ducts. Figure displays IHC staining for Mier3 as compared to IgG in transverse sections of rat mammary gland. Red arrows point to a rat mammary duct. The first panel shows an H&E stained section to observer the general layout of the tissue, the second panel shows the same section IHC stained for IgG, and the third panel shows this IHC stained section for Mier3.

Overall Goals

This goal of this dissertation work is two fold. The first goal will be to confirm the existence of predicted mammary carcinoma susceptibility QTL on *RN01* named *Mcs3.* If confirmed, this can potentially lead to the discovery of new mammary carcinoma susceptibility modifiers and related mechanisms. The second goal of this dissertation work will be to further characterize the *Mcs1b* locus. The *Mcs1b* locus can be delineated to a shorter region on chromosome *2* or a complex interaction in this region may be identified; either of these results will help identify susceptibility modifier mechanism. *Mier3* is either an ortholog of a candidate breast cancer susceptibility gene or a potential marker of a cell or tissue type that contributes to or explains altered mammary carcinoma susceptibility. Experiments proposed will help identify which of these roles *Mier3* plays in mammary carcinoma susceptibility.

Hypotheses

Hypotheses 1

Mcs3 QTL is an independently acting mammary carcinoma susceptibility locus on *RN01*. *Mcs3* may have at least two independently acting sub-loci corresponding to two peak linkage analysis markers within this region.

Hypotheses 2

Mcs1b is a complex locus which alters DMBA-induced mammary carcinoma susceptibility. Congenic line 1b-11 contains a genetic element/s responsible for the *Mcs1b* associated phenotypes.

Hypotheses 3

Differential mammary gland expression levels of *Mier3* between *Mcs1b* line N3 and susceptible WF females are due to a difference in MEC numbers between mammary glands of WF and Mcs1b congenic rats.

Specific Aims

These hypotheses will be tested by the following aims:

Specific Aim 1

Physically confirm predicted mammary carcinoma susceptibility QTL on *RN01* named *Mcs3* using WF.Cop congenic rats. I will simultaneously determine if, *Mcs3* is defined by two independently acting sub-loci.

Specific Aim 2

To determine if the WF.Cop line 1b-11 allele alters *Mcs1b* associated phenotypes.

Specific Aim 3

To determine if *Mier3* is differentially expressed due to mammary epithelial cell population differences between congenic N3 and WF rats.

To determine if *Mier3* levels are lower in mammary gland tissue of *Mcs1b* rats due to genotype associated differences in mammary gland epithelial cell numbers.

To determine if *Mier3* is differentially expressed in mammary gland tissue of *Mcs1b*-resistant and *Mcs1b* -susceptible rats due to a difference in *Mier3* expression levels in a population enriched for mammary gland epithelial cell.

CHAPTER II

MAMMARY CARCINOMA SUSCEPTIBILITY LOCUS, *MCS3*: PHYSICAL CONFIRMATION AND COMPARATIVE GENOMICS

Introduction

According to cancer statistics for 2016, breast cancer will be the most commonly diagnosed cancer among women, accounting for 29% of cancers diagnosed and 14% of cancer related deaths in women. Women have a population-based estimated 1 in 8 chance of being diagnosed with breast cancer in their lifetime [1]. It is a complex disease controlled by genetic, epigenetic and environmental factors. The genetic component of breast cancer susceptibility is comprised of high to low penetrance risk alleles [183]. Linkage analysis and genome-wide association studies have enabled the identification of several of these alleles, but a majority of these alleles remain to be identified [99, 184-186]. Emerging research is currently focusing on breast cancer not as a single disease, but as an array of diseases associated with varying mechanisms. Different approaches (e.g. cell culture studies, animal model based studies, and genome wide association studies) are being used to study breast cancer, each with its own advantages and disadvantages [157, 187, 188].

Technology has advanced leaps and bounds and sequencing costs have declined considerably making genome wide association studies (GWAS) a very popular method to address genetic variations associated with breast cancer. However, like any method,

GWAS have its limitations and drawbacks. One of the basic problems is identifying spurious associations caused due to confounding effects of environment and/or genetic background [189]. Another problem associated with GWAS is the use and comparison of heterogeneous populations. This not only leads to spurious associations, but also hinders the identification of single nucleotide variants (SNVs) with smaller effect sizes [190]. Associated risk alleles identified with GWAS may nominate candidate genes, but identifying the causal genes requires fine mapping and, molecular and mechanistic studies. These studies become especially challenging if the SNVs detected fall in gene deserts [191, 192]. One way these shortcomings of GWAS might be overcome is by studying breast cancer risk alleles using experimental organisms. Rats vary in susceptibility to mammary carcinogenesis. These rat models help identify loci associated with breast cancer which can be tested in human populations to identify SNVs associated with breast cancer risk [193]. The added advantage of using rats is that it offers an experimental model to study genetically determined mechanisms associated with breast cancer [194].

Inbred rat strains that differ in susceptibility to 7,12-Dimethylbenz[a]anthracene (*DMBA*) induced carcinogenesis have been used to identify mammary carcinoma susceptibility (Mcs) loci by linkage analysis [172, 173]. Rats are a good model of human breast cancer, as rat mammary carcinomas are similar to human breast carcinomas with respect to histopathology [195]. The cell of origin of both rat mammary and human breast carcinomas is mammary ductal cells [196]. Rat mammary carcinomas are also similar to human breast cancers with respect to hormone responsiveness; this is opposed to mouse mammary carcinomas, which are usually refractive to hormones [195]. Linkage analysis

using the DMBA sensitive Wistar-Furth (WF) strain and DMBA resistant Copenhagen (Cop) strain led to the prediction of four *Mcs* loci (i.e. *Mcs1, Mcs2, Mcs3,* and *Mcs4)* [172, 173]. In this study, we focused on *Mcs3,* a predicted *Mcs* QTL, with two peak markers, *D1Mit11* and *D1Wox6,* present on rat chromosome *1* [173]. We have physically confirmed *Mcs3* and mapped this QTL to a 25.8 MB region on rat chromosome *1*.

Results

Physical Confirmation and Fine Mapping *Mcs3* Using WF.Cop Congenic Rats

Figure 6 is a congenic map of WF.Cop congenic lines that were used to physically confirm and map the location of rat *Mcs3* QTL that was predicted by Gould and colleagues to exist on rat chromosome *1* [181]. Table 1 contains the DMBA induced mammary carcinoma susceptibility phenotypes (mean ± SD mammary tumor number per rat) of the congenic lines depicted in Figure 6. The WF.Cop congenic lines A and D developed 3.4 ± 2.2 and 2.8 ± 2.3 DMBA-tumors/rat, respectively. A combined group of susceptible WF females and WF/WF homozygous genotype littermates from congenic lines developed 6.3 ± 3.9 DMBA-induced tumors per rat. The susceptible WF phenotype in this study was similar to results previously published by denDekker *et al* where susceptible control WF rats developed 6.3 ± 3.3 tumors per rat upon DMBA induction [197]. Lines A and D rats developed significantly less mammary tumors per rat compared to the susceptible WF females (Mann-Whitney test *post hoc* p-values = 0.0208 and 0.0167, respectively). Reduced susceptibility lines A and D were not different from each other with respect to DMBA induced tumor multiplicity (Mann-Whitney test *post hoc* p-values = 0.496). Thus, the reduced susceptibility phenotypes of lines A and D physically confirmed the *Mcs3* locus.

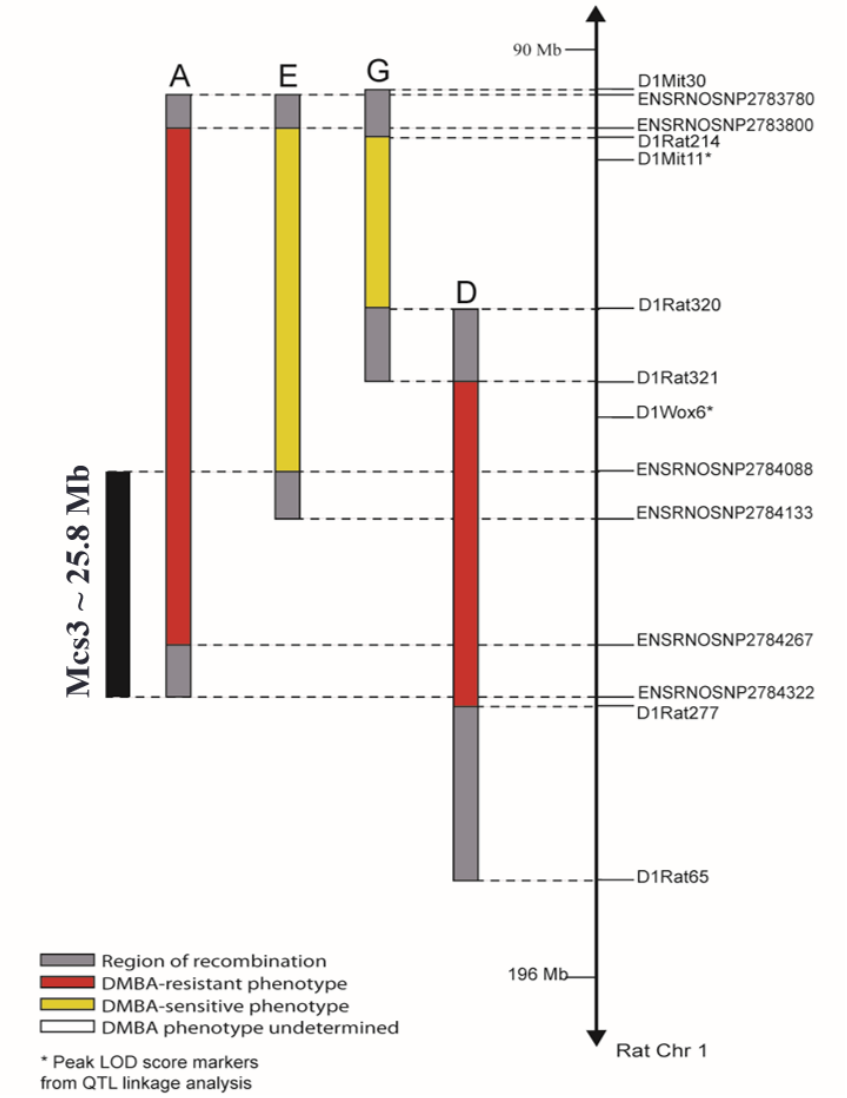


Figure 6. WF.Cop Congenic Map that Defines the *Mcs3* Locus. Figure represents varying segments on rat chromosome *1* where *Mcs3* was predicted to be located and congenic lines that were used to map this locus. On the axis, horizontal lines represent informative markers that were used to distinguish the WF genotype from the Cop genotype. Superscript letters indicate which line the respective line was derived from. Red bars represent Cop alleles in congenic lines with a resistant phenotype, yellow bars represent congenic lines with a susceptible phenotype, and grey bars represent segments of unknown recombination where the genotype wasn't determined.

Table 1. Summary of Mammary Carcinoma Susceptibility Phenotype of WF.Cop Congenic Lines Used to Map the *Mcs3* Locus

	WF.Cop congenic lines				
	A	D	E	G	WF/NHsd
Congenic region [1] (marker/marker)	ENSRNOSNP2783780/ ENSRNOSNP2784322	D1Rat320 / D1Rat65	ENSRNOSNP2783780/ ENSRNOSNP2784133	D1Mit30/ D1Rat321	-
Mean (SD) Mammary Carcinomas *per* Rat	3.4 (2.2)	2.8 (2.3)	6.6 (3.4)	8.1 (3.4)	6.3 (3.9)
N	30	19	25	29	12
p-value [2]	0.010	0.008	0.467	0.496	

[1]Informative markers that identified the length of the Cop Chr1 segment introgressed onto a susceptible WF genetic background.

[2]p-values are obtained by performing Mann-Whitney nonparametric *post hoc* tests and comparing each phenotype to control WF phenotype after a statistically significant Kruskal-Wallis test of p-value < 0.0125.

Congenic lines E and G developed 6.6 ± 3.4 and 8.1 ± 3.4 mammary tumors per rat, respectively. Lines E and G did not differ in tumor multiplicity compared to susceptible WF rats (Mann-Whitney test *post hoc* p-values = 0.467677 and 0.496 respectively). Lines E and G phenotypes were not different when compared with each other (Mann-Whitney test *post hoc* p-values = 0.496). The congenic segment overlap between lines that contain at least one independently acting allele (A and D), and the overlap and non-overlap of these lines with congenic line segments that do not contain independently acting alleles (E and G) can be used to delimit QTL intervals. Congenic *Mcs3*-asscociated reduced-susceptibility lines A and D overlapped each other minimally from markers *D1Rat321* to *ENSRNOSNP2784267,* and maximally from *D1Rat 320* to *ENSRNOSNP2784322* (Figure 6). Susceptible line E overlapped lines A and D from *ENSRNOSNP2783800* to *ENSRNOSNP2784088.* Susceptible Line G overlapped susceptible line A. These results indicated that the region between *ENSRNOSNP2783800* and *ENSRNOSNP2784088* does not contain an independently acting *Mcs* QTL. Taken together, our congenic studies here indicated lines A, D, E and G delimited the *Mcs3* QTL to a 25.8 Mb region on rat chromosome *1* that spans from *ENSRNOSNP2784088* to *ENSRNOSNP2784322* (~25.8 Mb, 138,096,182 – 163,886,079).

Human Ortholog to Rat *Mcs3* Locus

The *Mcs3* locus from *ENSRNOSNP2784088* to *ENSRNOSNP2784322* was found to align to four syntenic regions that are on human chromosomes 11 and 15 as shown in table 2. Figure 7 contains known genes at the *Mcs3* locus. These genes were identified using the November 2004 rat assembly (build 3.4/rn4) at the UCSC Genome Browser. The

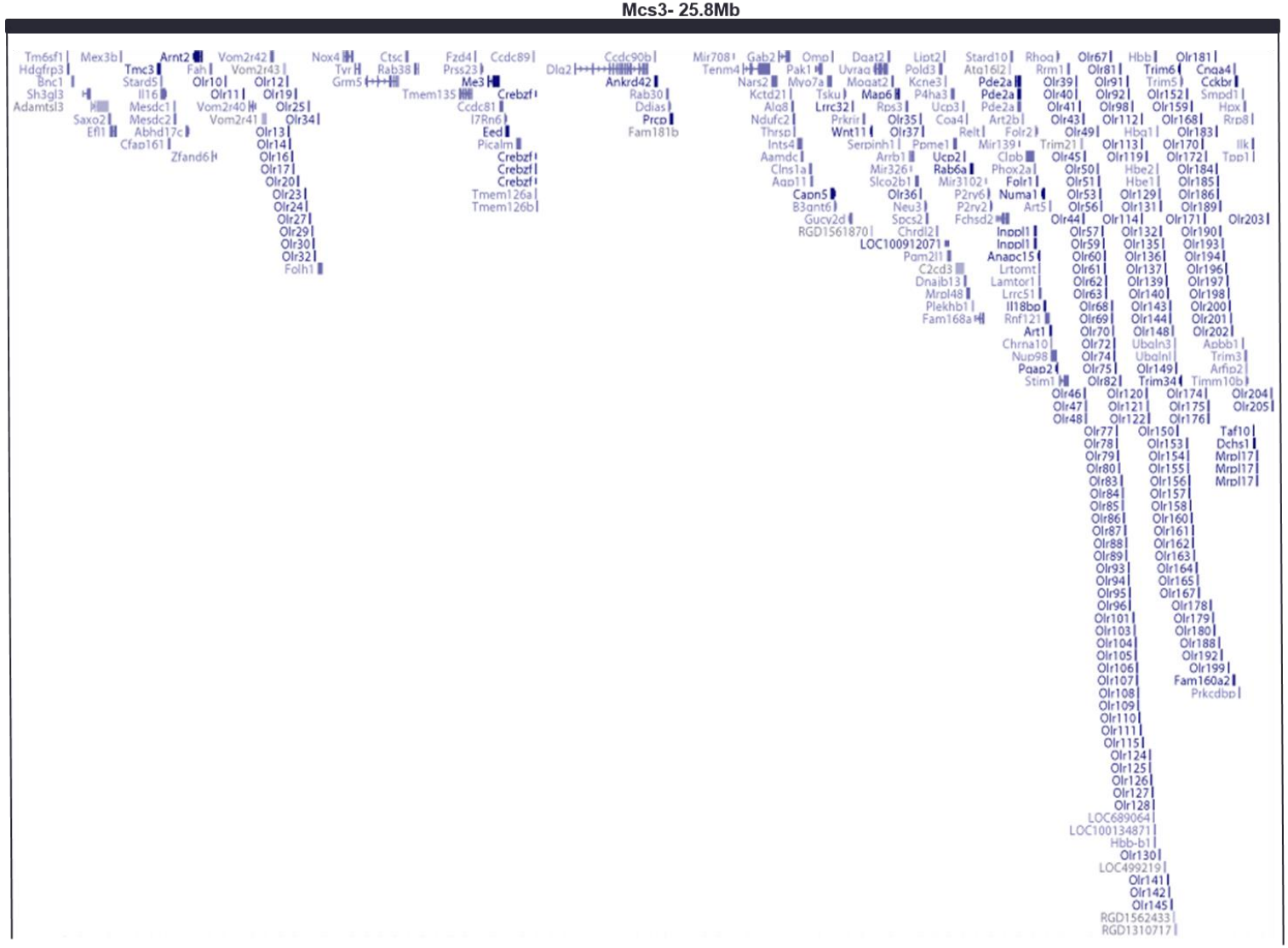

Mcs3- 25.8Mb

Figure 7. Transcript Map of the Defined *Mcs3* Locus. Map shows known and predicted transcripts within the defined *Mcs3* locus that were annotated on the UCSC *Rattus norvegicus* version 3.4/rn4 Genome Browser. The X axis represents the *Mcs3* locus which is present on rat chromosome 1 from genetic makers *D1 Rat350* to *D1 Rat277*. The marker position is represented by labeled tick marks on the X-axis. The vertical lines demarcate the maximal *Mcs3* locus. The transcripts are shown by their NCBI gene symbols at their approximate genomic positions.

Table 2. Loci on Human Chromosome That are Syntenic to the Rat *Mcs3* Locus on Chromosome *1* from *ENSRNOSNP2784088* to *ENSRNOSNP2784322*.

Information regarding human loci that are Orthologous to Rat *Mcs3* locus (Chromosome 1: 138,096,182 – 163,886,079)		
Human chromosome: base position	Percent identity between bases (%)	Percent identity between span (%)
11: 71,626,810 –89,350,902	25.3	63.3
11: 3,631,069 –6,744,074	2.9	15.7
15: 80,282,370 –82,577,745	3.6	8.2
15: 83,803,297 –84,806,774	1.3	3.4

Position is *Rattus norvegicus* genome build version 3.4.

Position is *Homo sapiens* genome build version hg19.

Table 3. List of Genes and Genomic Position in the Rat *Mcs3* Locus and in the Orthologous Human Region Obtained Using Ensembl Genome Browser

Rattus norvegicus		Homo sapiens	
Genes	Location	Homologues	Location
Hdgfrp3 (ENSRNOG00000019740)	1:138098819-138119696	RP11-382A20.3 (ENSG00000166503)	15:83784320-83876770
D4A0X9_RAT (ENSRNOG00000019770)	1:138189345-138202803	BNC1 (ENSG00000169594)	15:83924655-83953466
Sh3gl3 (ENSRNOG00000019776)	1:138398334-138529056	SH3GL3 (ENSG00000140600)	15:84115980-84287495
F1M767_RAT (ENSRNOG00000010840)	1:138563870-138876886	ADAMTSL3 (ENSG00000156218)	15:84322838-84708594
Fam154b (ENSRNOG00000026296)	1:138905988-138920522	FAM154B (ENSG00000188659)	15:82555151-82577271
Eftud1 (ENSRNOG00000032723)	1:138920620-139014913	EFTUD1 (ENSG00000140598)	15:82422571-82555104
D3ZFL6_RAT (ENSRNOG00000025142)	1:139135884-139138449	MEX3B (ENSG00000183496)	15:82334119-82338482
D4ABU9_RAT (ENSRNOG00000036972)	1:139418553-139442493	No homologues	
Tmc3 (ENSRNOG00000025088)	1:139842574-139886756	TMC3 (ENSG00000188869)	15:81623558-81666554
Stard5 (ENSRNOG00000025052)	1:139896272-139905625	STARD5 (ENSG00000172345)	15:81601394-81616524
Il16 (ENSRNOG00000011680)	1:139908061-140000146	IL16 (ENSG00000172349)	15:81451916-81605104
RGD1310371 (ENSRNOG00000012301)	1:140036328-140061890	C15orf26 (ENSG00000156206)	15:81299374-81441516
Mesdc1 (ENSRNOG00000012349)	1:140157974-140159062	MESDC1 (ENSG00000140406)	15:81293295-81296342
Mesdc2 (ENSRNOG00000012366)	1:140167851-140173514	MESDC2 (ENSG00000117899)	15:81239667-81282219
RGD1305254 (ENSRNOG00000012442)	1:140203040-140270662	KIAA1199 (ENSG00000103888)	15:81071684-81244117
Fam108c1 (ENSRNOG00000012683)	1:140382431-140423393	FAM108C1 (ENSG00000136379)	15:80972025-81047962
Arnt2 (ENSRNOG00000013017)	1:140535823-140693148	ARNT2 (ENSG00000172379)	15:80696692-80890278
FAAA_RAT (ENSRNOG00000013223)	1:140851979-140876087	FAH (ENSG00000103876)	15:80444832-80479288
Zfand6 (ENSRNOG00000013506)	1:140885729-140960486	ZFAND6 (ENSG00000086666)	15:80351910-80430735
Olr10 (ENSRNOG00000029100)	1:141151954-141152901	No homologues	
Olr11 (ENSRNOG00000032046)	1:141497719-141498669	No homologues	
Vom2r40 (ENSRNOG00000030229)	1:141602289-141721711	No homologues	
Vom2r41 (ENSRNOG00000028804)	1:141861806-141914331	No homologues	
Vom2r42 (ENSRNOG00000043159)	1:142010227-142055405	No homologues	
Vom2r43 (ENSRNOG00000013563)	1:142267794-142290322	No homologues	
Olr12 (ENSRNOG00000033009)	1:142339329-142340252	OR13G1 (ENSG00000197437)	1:247835320-247836365
Olr13 (ENSRNOG00000032371)	1:142363442-142364368	OR6F1 (ENSG00000169214)	1:247875045-247876105
Olr14 (ENSRNOG00000030565)	1:142393184-142394125	OR14A2 (ENSG00000241128)	1:247886401-247887345
Olr16 (ENSRNOG00000031741)	1:142435591-142436541	OR14A2 (ENSG00000241128)	1:247886401-247887345
Olr17 (ENSRNOG00000030774)	1:142443502-142444458	No homologues	
Olr19 (ENSRNOG00000032077)	1:142491573-142492532	No homologues	
D4A8P2_RAT (ENSRNOG00000024653)	1:142500803-142501792	No homologues	
Olr20 (ENSRNOG00000032843)	1:142539267-142540259	No homologues	
Olr23 (ENSRNOG00000029907)	1:142677366-142678361	No homologues	
Olr24 (ENSRNOG00000031858)	1:142710362-142711303	OR14A2 (ENSG00000241128)	1:247886401-247887345
Olr25 (ENSRNOG00000028897)	1:142740112-142741104	No homologues	
Olr27 (ENSRNOG00000033105)	1:142782825-142783766	OR14A2 (ENSG00000241128)	1:247886401-247887345
D3ZPM8_RAT (ENSRNOG00000032179)	1:142795102-142800862	No homologues	
Olr29 (ENSRNOG00000030176)	1:142819617-142820624	OR14A2 (ENSG00000241128)	1:247886401-247887345
Olr30 (ENSRNOG00000029233)	1:142835594-142836523	No homologues	
Olr32 (ENSRNOG00000033463)	1:142871429-142872436	OR14A2 (ENSG00000241128)	1:247886401-247887345
Olr34 (ENSRNOG00000030554)	1:142922930-142923868	No homologues	
Folh1 (ENSRNOG00000013770)	1:142936379-143010358	FOLH1 (ENSG00000086205)	11:49168187-49230222
F1M627_RAT (ENSRNOG00000021928)	1:142990978-142991758	No homologues	
LOC682236 (ENSRNOG00000033507)	1:143105977-143137062	No homologues	
F1M7J6_RAT (ENSRNOG00000028905)	1:143224045-143225319	No homologues	
Nox4 (ENSRNOG00000013925)	1:143415816-143603546	NOX4 (ENSG00000086991)	11:89057524-89322779
Tyr (ENSRNOG00000016421)	1:143641257-143746315	TYR (ENSG00000077498)	11:88910620-89028908
Grm5 (ENSRNOG00000016429)	1:143863506-144477283	GRM5 (ENSG00000168959)	11:88237744-88799113
Ctsc (ENSRNOG00000016496)	1:144629802-144661183	CTSC (ENSG00000109861)	11:88026773-88070955
Rab38 (ENSRNOG00000016769)	1:144783909-144864574	RAB38 (ENSG00000123892)	11:87846431-87908635
F1M7E7_RAT (ENSRNOG00000031869)	1:144968576-144968944	No homologues	
Tmem135 (ENSRNOG00000016815)	1:145664797-145894704	TMEM135 (ENSG00000166575)	11:86748886-87034756
Fzd4 (ENSRNOG00000016848)	1:145953746-145957666	FZD4 (ENSG00000174804)	11:86656721-86666433
Prss23 (ENSRNOG00000017307)	1:146081557-146089928	PRSS23 (ENSG00000150687)	11:86502101-86663886
F1M6N3_RAT (ENSRNOG00000017296)	1:146082484-146082870	No homologues	
F1LUQ4_RAT (ENSRNOG00000017311)	1:146313072-146422586	ME3 (ENSG00000151376)	11:86152150-86383678
D4A6D5_RAT (ENSRNOG00000031186)	1:146425722-146426346	No homologues	

Table 3. List of Genes and Genomic Position in the Rat *Mcs3* Locus and in the Orthologous Human Region Obtained Using Ensembl Genome Browser (continued)

Rattus norvegicus		Homo sapiens	
Genes	Location	Homologues	Location
D4ADM4_RAT (ENSRNOG00000033733)	1:146430328-146487051	CCDC81 (ENSG00000149201)	11:86085778-86134151
LOC680217 (ENSRNOG00000022941)	1:146506681-146507085	No homologues	
LOC293103 (ENSRNOG00000017383)	1:146522015-146545246	C11orf73 (ENSG00000149196)	11:86013253-86056969
Eed (ENSRNOG00000017509)	1:146563964-146591042	EED (ENSG00000074266)	11:85955586-85989781
Picalm (ENSRNOG00000018322)	1:146754045-146834296	PICALM (ENSG00000073921)	11:85668727-85780924
LOC680270 (ENSRNOG00000022808)	1:146869691-146919120	CCDC83 (ENSG00000150676)	11:85566144-85631064
F1LN23_RAT (ENSRNOG00000030776)	1:146977959-147084664	SYTL2 (ENSG00000137501)	11:85405267-85522184
Ccdc89 (ENSRNOG00000022766)	1:147091688-147092949	CCDC89 (ENSG00000179071)	11:85394893-85397320
Crebzf (ENSRNOG00000018987)	1:147124224-147129115	CREBZF (ENSG00000137504)	11:85370752-85393951
Tmem126a (ENSRNOG00000022748)	1:147129335-147137259	TMEM126A (ENSG00000171202)	11:85359011-85367591
Tmem126b (ENSRNOG00000022732)	1:147146187-147158120	TMEM126B (ENSG00000171204)	11:85339629-85347580
Dlg2 (ENSRNOG00000022635)	1:148051658-149274633	DLG2 (ENSG00000150672)	11:83166055-85338966
F1LZX0_RAT (ENSRNOG00000033242)	1:149145746-149173468	No homologues	
F1M2B1_RAT (ENSRNOG00000022627)	1:149355092-149356667	RP11-574F21.3 (ENSG00000258465)	1:160187254-160254920
		DCAF8 (ENSG00000132716)	1:160185505-160254920
Ccdc90b (ENSRNOG00000009462)	1:149410497-149424047	CCDC90B (ENSG00000137500)	11:82970139-82997450
Ankrd42 (ENSRNOG00000009664)	1:149423846-149468863	ANKRD42 (ENSG00000137494)	11:82904754-82971736
Pcf11 (ENSRNOG00000009891)	1:149475611-149501027	PCF11 (ENSG00000165494)	11:82868030-82898493
Rab30 (ENSRNOG00000010224)	1:149656373-149670208	RAB30 (ENSG00000137502)	11:82684175-82782965
RGD1559690 (ENSRNOG00000022521)	1:149685958-149710264	C11orf82 (ENSG00000165490)	11:82611017-82669319
Prcp (ENSRNOG00000010630)	1:149711289-149763868	PRCP (ENSG00000137509)	11:82534544-82681626
F1M1I2_RAT (ENSRNOG00000023369)	1:149785072-149786082	No homologues	
Fam181b (ENSRNOG00000010763)	1:149955641-149957129	FAM181B (ENSG00000182103)	11:82443053-82444906
RGD1561957 (ENSRNOG00000032343)	1:151354250-151354732	No homologues	
D4A3T7_RAT (ENSRNOG00000025368)	1:153438818-153439081	No homologues	
Odz4 (ENSRNOG00000011151)	1:153808625-154172717	ODZ4 (ENSG00000149256)	11:78363876-79151992
Nars2 (ENSRNOG00000011476)	1:154216134-154329582	NARS2 (ENSG00000137513)	11:78147007-78285919
Gab2 (ENSRNOG00000011882)	1:154348781-154544906	GAB2 (ENSG00000033327)	11:77926343-78129394
D4AAA3_RAT (ENSRNOG00000024961)	1:154549903-154565904	USP35 (ENSG00000118369)	11:77899858-77925757
Kctd21 (ENSRNOG00000024793)	1:154587686-154588566	KCTD21 (ENSG00000188997)	11:77882295-77899868
Alg8 (ENSRNOG00000012292)	1:154607442-154628226	ALG8 (ENSG00000159063)	11:77811982-77850706
Ndufc2 (ENSRNOG00000012383)	1:154635889-154642111	NDUFC2 (ENSG00000151366)	11:77779350-77791265
		NDUFC2-KCTD14 (ENSG00000259112	11:77728017-77790911
Thrsp (ENSRNOG00000012404)	1:154647341-154651663	THRSP (ENSG00000151365)	11:77774907-77779397
D4AAA7_RAT (ENSRNOG00000036873)	1:154660657-154661255	PRDX2 (ENSG00000167815)	19:12907634-12912694
Kctd14 (ENSRNOG00000012494)	1:154684785-154690800	KCTD14 (ENSG00000151364)	11:77726761-77757237
D3ZZQ6_RAT (ENSRNOG00000012552)	1:154714605-154778361	INTS4 (ENSG00000149262)	11:77589766-77705724
RGD1561459 (ENSRNOG00000012584)	1:154786525-154800839	C11orf67 (ENSG00000087884)	11:77532155-77629478
D3ZGQ8_RAT (ENSRNOG00000024194)	1:154817662-154935717	RSF1 (ENSG00000048649)	11:77371041-77532063
Clns1a (ENSRNOG00000012788)	1:154946673-154966945	CLNS1A (ENSG00000074201)	11:77225981-77348850
Aqp11 (ENSRNOG00000013358)	1:154973798-154983989	AQP11 (ENSG00000178301)	11:77300436-77321400
Pak1 (ENSRNOG00000029784)	1:155057622-155174708	PAK1 (ENSG00000149269)	11:77032752-77185680
F1M812_RAT (ENSRNOG00000028470)	1:155199289-155253342	GDPD4 (ENSG00000178795)	11:76927603-77012732
D4AB24_RAT (ENSRNOG00000013641)	1:155292620-155362698	MYO7A (ENSG00000137474)	11:76839310-76926284
Capn5 (ENSRNOG00000014251)	1:155366703-155421183	CAPN5 (ENSG00000149260)	11:76777979-76837201
B3gnt6 (ENSRNOG00000014471)	1:155435459-155440490	B3GNT6 (ENSG00000198488)	11:76745385-76753096
Acer3 (ENSRNOG00000036866)	1:155455466-155509236	ACER3 (ENSG00000078124)	11:76571911-76737841
Tsku (ENSRNOG00000027784)	1:155611070-155622378	TSKU (ENSG00000182704)	11:76493295-76509198
F1M245_RAT (ENSRNOG00000015058)	1:155716478-155747851	No homologues	
Lrrc32 (ENSRNOG00000015310)	1:155761334-155771294	LRRC32 (ENSG00000137507)	11:76368568-76381791
D4AC97_RAT (ENSRNOG00000015560)	1:155862045-155931127	C11orf30 (ENSG00000158636)	11:76155967-76264069
Prkrir (ENSRNOG00000015808)	1:155982782-155997548	PRKRIR (ENSG00000137492)	11:76061000-76092015
Wnt11 (ENSRNOG00000015982)	1:156126223-156140802	WNT11 (ENSG00000085741)	11:75897369-75921803
RGD1561870 (ENSRNOG00000042782)	1:156142284-156142592	No homologues	
D3ZKE1_RAT (ENSRNOG00000016206)	1:156174285-156435696	UVRAG (ENSG00000198382)	11:75526212-75854239
Dgat2 (ENSRNOG00000016573)	1:156447588-156478740	DGAT2 (ENSG00000062282)	11:75470557-75512579
Mogat2 (ENSRNOG00000027228)	1:156516533-156540899	MOGAT2 (ENSG00000166391)	11:75428864-75444003
Map6 (ENSRNOG00000027204)	1:156592504-156657997	MAP6 (ENSG00000171533)	11:75297963-75380165

Table 3. List of Genes and Genomic Position in the Rat *Mcs3* Locus and in the Orthologous Human Region Obtained Using Ensembl Genome Browser (continued)

Rattus norvegicus		Homo sapiens	
Genes	Location	Homologues	Location
Serpinh1 (ENSRNOG00000016831)	1:156667042-156674221	SERPINH1 (ENSG00000149257)	11:75273101-75283828
B5DF39_RAT (ENSRNOG00000036859)	1:156703241-156794530	GDPD5 (ENSG00000158555)	11:75145685-75236948
Klhl35 (ENSRNOG00000017365)	1:156801077-156806657	KLHL35 (ENSG00000149243)	11:75133438-75141674
Rps3 (ENSRNOG00000017418)	1:156811473-156816770	No homologues	
Arrb1 (ENSRNOG00000030404)	1:156871562-156937540	ARRB1 (ENSG00000137486)	11:74975226-75062873
LOC681316 (ENSRNOG00000017922)	1:156958285-156959444	No homologues	
Slco2b1 (ENSRNOG00000017976)	1:156992493-157028887	SLCO2B1 (ENSG00000137491)	11:74811608-74917594
Q5RJS1_RAT (ENSRNOG00000018071)	1:157074498-157076449	OR2AT4 (ENSG00000171561)	11:74799758-74800799
Olr36 (ENSRNOG00000030788)	1:157085954-157086934	No homologues	
Olr37 (ENSRNOG00000018078)	1:157123043-157124008	No homologues	
Neu3 (ENSRNOG00000018106)	1:157172548-157183064	NEU3 (ENSG00000162139)	11:74699179-74729938
Spcs2 (ENSRNOG00000018164)	1:157199447-157217395	SPCS2 (ENSG00000118363)	11:74660292-74690076
F1MAJ1_RAT (ENSRNOG00000026503)	1:157217625-157281411	XRRA1 (ENSG00000166435)	11:74518784-74660245
LOC685009 (ENSRNOG00000026408)	1:157289186-157313991	RNF169 (ENSG00000166439)	11:74459913-74553458
LOC100360934 (ENSRNOG00000018394)	1:157391482-157399282	CHRDL2 (ENSG00000054938)	11:74407474-74442430
Pold3 (ENSRNOG00000018411)	1:157453059-157490563	POLD3 (ENSG00000077514)	11:74204896-74380162
Lipt2 (ENSRNOG00000016906)	1:157538704-157541031	LIPT2 (ENSG00000175536)	11:74202757-74204778
Kcne3 (ENSRNOG00000017054)	1:157558044-157565007	KCNE3 (ENSG00000175538)	11:74165886-74178774
Pgm2l1 (ENSRNOG00000017079)	1:157605906-157655227	PGM2L1 (ENSG00000165434)	11:74041363-74109518
P4ha3 (ENSRNOG00000017118)	1:157663338-157720713	P4HA3 (ENSG00000149380)	11:73946846-74022702
Ppme1 (ENSRNOG00000017227)	1:157725633-157773879	PPME1 (ENSG00000214517)	11:73882144-73965748
D3ZXD6_RAT (ENSRNOG00000017608)	1:157774329-157892686	C2CD3 (ENSG00000168014)	11:73723763-73882255
Ucp3 (ENSRNOG00000017716)	1:157896001-157909606	UCP3 (ENSG00000175564)	11:73711326-73720480
Ucp2 (ENSRNOG00000017854)	1:157922179-157928518	UCP2 (ENSG00000175567)	11:73685712-73694352
Dnajb13 (ENSRNOG00000017975)	1:157931501-157945870	DNAJB13 (ENSG00000187726)	11:73661364-73681411
Chchd8 (ENSRNOG00000025385)	1:157965119-157968347	CHCHD8 (ENSG00000181924)	11:73583712-73588033
Mrpl48 (ENSRNOG00000018042)	1:157979818-158022405	MRPL48 (ENSG00000175581)	11:73498361-73576178
Rab6a (ENSRNOG00000018176)	1:158041088-158080213	RAB6A (ENSG00000175582)	11:73386938-73472182
		RAB6C (ENSG00000222014)	2:130737235-130740311
Plekhb1 (ENSRNOG00000018627)	1:158081930-158099517	PLEKHB1 (ENSG00000021300)	11:73357223-73373864
Fam168a (ENSRNOG00000018873)	1:158140473-158287606	FAM168A (ENSG00000054965)	11:73111532-73309234
Relt (ENSRNOG00000025075)	1:158293361-158300581	RELT (ENSG00000054967)	11:73087309-73108519
D4ADZ1_RAT (ENSRNOG00000019192)	1:158316868-158375737	ARHGEF17 (ENSG00000110237)	11:73019334-73080136
P2ry6 (ENSRNOG00000019270)	1:158381128-158406783	P2RY6 (ENSG00000171631)	11:72975550-73009662
P2ry2 (ENSRNOG00000019283)	1:158440062-158446040	P2RY2 (ENSG00000175591)	11:72929343-72947397
Fchsd2 (ENSRNOG00000019319)	1:158548830-158782422	FCHSD2 (ENSG00000137478)	11:72547790-72853306
Q6QI52_RAT (ENSRNOG00000033196)	1:158590389-158593205	No homologues	
Atg16l2 (ENSRNOG00000019413)	1:158787779-158800330	ATG16L2 (ENSG00000168010)	11:72525353-72554719
Stard10 (ENSRNOG00000019491)	1:158819970-158845080	STARD10 (ENSG00000214530)	11:72465774-72505213
F1LM60_RAT (ENSRNOG00000019555)	1:158846702-158912163	ARAP1 (ENSG00000186635)	11:72396114-72504644
Pde2a (ENSRNOG00000019560)	1:158921607-159013829	PDE2A (ENSG00000186642)	11:72287185-72385635
Art2 (ENSRNOG00000019687)	1:159055461-159058780	No homologues	
F1LXX4_RAT (ENSRNOG00000042674)	1:159116498-159117496	No homologues	
Clpb (ENSRNOG00000019693)	1:159126512-159246279	CLPB (ENSG00000162129)	11:72003469-72145692
Phox2a (ENSRNOG00000019706)	1:159273524-159277731	PHOX2A (ENSG00000165462)	11:71950121-71956708
Inppl1 (ENSRNOG00000019730)	1:159278130-159291910	INPPL1 (ENSG00000165458)	11:71934745-71950149
Folr2 (ENSRNOG00000019890)	1:159295773-159300030	FOLR2 (ENSG00000165457)	11:71927645-71932994
Folr1 (ENSRNOG00000019902)	1:159315192-159324141	FOLR1 (ENSG00000110195)	11:71900602-71907345
RGD1311634 (ENSRNOG00000019936)	1:159359110-159362843	C11orf51 (ENSG00000110200)	11:71817424-71823826
Lrtomt (ENSRNOG00000023434)	1:159362010-159364059	LRTOMT (ENSG00000184154)	11:71791382-71821828
MGC72560 (ENSRNOG00000020016)	1:159367471-159373042	LAMTOR1 (ENSG00000149357)	11:71796941-71814433
Lrrc51 (ENSRNOG00000020124)	1:159373973-159393171	No homologues	
F1LW91_RAT (ENSRNOG00000000417)	1:159435751-159470959	NUMA1 (ENSG00000137497)	11:71713910-71791739
Il18bp (ENSRNOG00000020150)	1:159471809-159473288	IL18BP (ENSG00000137496)	11:71709587-71716761
Rnf121 (ENSRNOG00000020175)	1:159475046-159542451	RNF121 (ENSG00000137522)	11:71639747-71708643
TRPC2_RAT (ENSRNOG00000020188)	1:159542820-159576982	No homologues	
F1M3Z1_RAT (ENSRNOG00000036817)	1:159546441-159547441	No homologues	
Art5 (ENSRNOG00000020242)	1:159576991-159586949	ART5 (ENSG00000167311)	11:3659733-3663546

Table 3. List of Genes and Genomic Position in the Rat *Mcs3* Locus and in the Orthologous Human Region Obtained Using Ensembl Genome Browser (continued)

Rattus norvegicus		Homo sapiens	
Genes	Location	Homologues	Location
Art1 (ENSRNOG00000020251)	1:159586246-159590797	ART1 (ENSG00000129744)	11:3666358-3685646
Chrna10 (ENSRNOG00000020293)	1:159590904-159595101	CHRNA10 (ENSG00000129749)	11:3686817-3692614
Nup98 (ENSRNOG00000020347)	1:159598048-159696720	NUP98 (ENSG00000110713)	11:3692313-3819022
Pgap2 (ENSRNOG00000020371)	1:159696920-159722558	PGAP2 (ENSG00000148985)	11:3818954-3847601
Rhog (ENSRNOG00000020393)	1:159724016-159735532	RHOG (ENSG00000177105)	11:3848208-3862213
Stim1 (ENSRNOG00000020425)	1:159761567-159925836	STIM1 (ENSG00000167323)	11:3875757-4114439
Olr40 (ENSRNOG00000018541)	1:160090914-160091975	No homologues	
Olr41 (ENSRNOG00000012071)	1:160145884-160146825	OR52B4 (ENSG00000221996)	11:4388493-4389616
Trim21 (ENSRNOG00000018517)	1:160175882-160187661	TRIM21 (ENSG00000132109)	11:4406127-4414926
Olr43 (ENSRNOG00000018514)	1:160208359-160209309	No homologues	
Olr44 (ENSRNOG00000018512)	1:160220248-160221195	No homologues	
Olr45 (ENSRNOG00000018508)	1:160237160-160238113	OR52K2 (ENSG00000181963)	11:4470525-4471591
Olr46 (ENSRNOG00000018506)	1:160247696-160248640	OR52K1 (ENSG00000196778)	11:4510109-4511138
LOC293190 (ENSRNOG00000018503)	1:160254067-160254579	BCL2L1 (ENSG00000171552)	20:30252255-30311792
Olr47 (ENSRNOG00000018501)	1:160258278-160259237	No homologues	
Olr48 (ENSRNOG00000018497)	1:160275366-160276438	OR52M1 (ENSG00000197790)	11:4566421-4567374
Olr49 (ENSRNOG00000015444)	1:160489681-160490646	OR51S1 (ENSG00000176922)	11:4869427-4870514
D3ZYR2_RAT (ENSRNOG00000015432)	1:160503871-160527438	OR51F1 (ENSG00000188069)	11:4790209-4791168
Olr51 (ENSRNOG00000015424)	1:160512753-160513703	OR51F2 (ENSG00000176925)	11:4842551-4843686
Olr53 (ENSRNOG00000031110)	1:160547823-160548767	No homologues	
Olr56 (ENSRNOG00000015412)	1:160573393-160574343	OR51C1P (ENSG00000197674)	11:4711988-4713394
Olr57 (ENSRNOG00000015407)	1:160580940-160581884	AC103710.1 (ENSG00000197426)	11:4730835-4731698
Olr59 (ENSRNOG00000018606)	1:160607895-160613802	OR51E2 (ENSG00000167332)	11:4701401-4719084
Olr60 (ENSRNOG00000029206)	1:160626353-160627315	No homologues	
Olr61 (ENSRNOG00000033046)	1:160634724-160635686	No homologues	
Olr62 (ENSRNOG00000031787)	1:160643870-160644832	No homologues	
Olr63 (ENSRNOG00000018551)	1:160647966-160648919	OR51E1 (ENSG00000180785)	11:4664650-4676718
Olr67 (ENSRNOG00000034083)	1:160746318-160747394	OR51A7 (ENSG00000176895)	11:4928600-4965356
Olr68 (ENSRNOG00000015649)	1:160765772-160766740	OR51A7 (ENSG00000176895)	11:4928600-4965356
Olr69 (ENSRNOG00000015657)	1:160771342-160773093	OR51G2 (ENSG00000176893)	11:4935900-4936922
Olr70 (ENSRNOG00000015673)	1:160788683-160789624	OR51G1 (ENSG00000176879)	11:4944531-4945637
F1LWR7_RAT (ENSRNOG00000015681)	1:160805014-160813315	No homologues	
Olr74 (ENSRNOG00000015689)	1:160825660-160826619	OR52R1 (ENSG00000176937)	11:4824663-4825847
Olr75 (ENSRNOG00000030860)	1:160833798-160834757	OR51F1 (ENSG00000188069)	11:4790209-4791168
Olr77 (ENSRNOG00000029270)	1:160853088-160854047	OR51F1 (ENSG00000188069)	11:4790209-4791168
Olr78 (ENSRNOG00000015760)	1:160866763-160867710	OR51L1 (ENSG00000176798)	11:5020213-5021160
Olr79 (ENSRNOG00000015764)	1:160873701-160874642	No homologues	
Olr80 (ENSRNOG00000015769)	1:160911661-160912676	OR52E2 (ENSG00000176787)	11:5079880-5080857
Olr81 (ENSRNOG00000028970)	1:160922554-160923492	OR52J3 (ENSG00000205495)	11:5067756-5068691
Olr82 (ENSRNOG00000033200)	1:160937940-160938884	No homologues	
Olr83 (ENSRNOG00000031586)	1:160955735-160956673	OR52J3 (ENSG00000205495)	11:5067756-5068691
Olr84 (ENSRNOG00000030275)	1:160970408-160971391	No homologues	
Olr85 (ENSRNOG00000015785)	1:160977271-160978206	No homologues	
Olr86 (ENSRNOG00000021332)	1:161007450-161008388	No homologues	
Olr87 (ENSRNOG00000021290)	1:161019428-161020366	No homologues	
Olr91 (ENSRNOG00000015804)	1:161076388-161077344	No homologues	
Olr92 (ENSRNOG00000015811)	1:161084121-161085065	No homologues	
Olr93 (ENSRNOG00000015819)	1:161092784-161093728	No homologues	
Olr94 (ENSRNOG00000036811)	1:161099003-161099974	No homologues	
Olr95 (ENSRNOG00000036810)	1:161107309-161108253	No homologues	
Olr96 (ENSRNOG00000015832)	1:161117890-161118846	No homologues	
Olr97 (ENSRNOG00000011001)	1:161128788-161130043	No homologues	
Olr98 (ENSRNOG00000033060)	1:161149322-161150269	No homologues	
F1LUF0_RAT (ENSRNOG00000043307)	1:161165381-161185205	No homologues	
Olr101 (ENSRNOG00000031820)	1:161175882-161176832	No homologues	
Olr103 (ENSRNOG00000030169)	1:161204913-161205863	No homologues	
Olr104 (ENSRNOG00000015876)	1:161216105-161217064	No homologues	
Olr105 (ENSRNOG00000015885)	1:161229983-161230930	No homologues	

Table 3. List of Genes and Genomic Position in the Rat *Mcs3* Locus and in the Orthologous Human Region Obtained Using Ensembl Genome Browser (continued)

Rattus norvegicus		Homo sapiens	
Genes	Location	Homologues	Location
Olr106 (ENSRNOG00000015893)	1:161240522-161241493	No homologues	
Olr107 (ENSRNOG00000015901)	1:161253006-161254085	No homologues	
Olr108 (ENSRNOG00000015909)	1:161270136-161271077	No homologues	
Olr109 (ENSRNOG00000015919)	1:161273981-161274934	No homologues	
Olr110 (ENSRNOG00000015924)	1:161286481-161287446	No homologues	
Olr111 (ENSRNOG00000015926)	1:161318671-161319618	No homologues	
Olr112 (ENSRNOG00000015933)	1:161330800-161331762	No homologues	
Olr113 (ENSRNOG00000015940)	1:161340241-161341182	No homologues	
Olr114 (ENSRNOG00000032357)	1:161347591-161348535	No homologues	
Olr115 (ENSRNOG00000031878)	1:161362778-161363731	No homologues	
F1M0A2_RAT (ENSRNOG00000015966)	1:161377757-161378526	NRBF2 (ENSG00000148572)	10:64893050-64914783
D3ZMJ6_RAT (ENSRNOG00000042552)	1:161418106-161419072	No homologues	
Olr119 (ENSRNOG00000033350)	1:161432556-161433509	OR51B6 (ENSG00000176239)	11:5344961-5373676
Olr120 (ENSRNOG00000031550)	1:161440488-161441432	No homologues	
Olr121 (ENSRNOG00000030395)	1:161457068-161458021	No homologues	
Olr122 (ENSRNOG00000029978)	1:161468297-161469247	No homologues	
F1M2P3_RAT (ENSRNOG00000032984)	1:161482950-161483890	No homologues	
Olr124 (ENSRNOG00000016018)	1:161490750-161491697	No homologues	
Olr125 (ENSRNOG00000016027)	1:161506536-161507495	No homologues	
Olr126 (ENSRNOG00000029612)	1:161520711-161521661	OR52A5 (ENSG00000171944)	11:5152922-5153872
Olr127 (ENSRNOG00000034039)	1:161542787-161543734	OR52A5 (ENSG00000171944)	11:5152922-5153872
Olr128 (ENSRNOG00000016052)	1:161566055-161567017	No homologues	
LOC689064 (ENSRNOG00000031230)	1:161578128-161579492	HBD (ENSG00000223609)	11:5253908-5256600
		HBB (ENSG00000244734)	11:5246694-5250625
Hbb (ENSRNOG00000033465)	1:161584858-161620198	HBD (ENSG00000223609)	11:5253908-5256600
		HBB (ENSG00000244734)	11:5246694-5250625
Hbb-b1 (ENSRNOG00000025510)	1:161590671-161598127	HBD (ENSG00000223609)	11:5253908-5256600
		HBB (ENSG00000244734)	11:5246694-5250625
Hbg1 (ENSRNOG00000030879)	1:161640240-161641771	No homologues	
Hbe2 (ENSRNOG00000030784)	1:161647651-161649279	No homologues	
Hbe1 (ENSRNOG00000029286)	1:161651306-161652708	HBE1 (ENSG00000213931)	11:5289582-5526847
Olr129 (ENSRNOG00000025163)	1:161677418-161678353	No homologues	
Olr130 (ENSRNOG00000031983)	1:161687005-161687928	No homologues	
Olr131 (ENSRNOG00000030179)	1:161697243-161698166	No homologues	
Olr132 (ENSRNOG00000029051)	1:161717426-161718373	OR51B6 (ENSG00000176239)	11:5344961-5373676
Olr135 (ENSRNOG00000043392)	1:161758920-161759858	No homologues	
Olr136 (ENSRNOG00000024877)	1:161771509-161772462	OR51J1 (ENSG00000184321)	11:5423827-5424777
LOC499219 (ENSRNOG00000024789)	1:161788384-161794974	No homologues	
Olr137 (ENSRNOG00000034041)	1:161796236-161797201	OR51Q1 (ENSG00000167360)	11:5443341-5444436
Olr139 (ENSRNOG00000031147)	1:161825890-161826855	OR51Q1 (ENSG00000167360)	11:5443341-5444436
Olr141 (ENSRNOG00000016946)	1:161856623-161857573	No homologues	
Olr142 (ENSRNOG00000016954)	1:161872355-161873299	OR51I1 (ENSG00000167359)	11:5451898-5489943
Olr143 (ENSRNOG00000016964)	1:161883447-161884385	OR51I2 (ENSG00000187918)	11:5474638-5475707
Olr144 (ENSRNOG00000016979)	1:161890517-161891461	No homologues	
F1LZ60_RAT (ENSRNOG00000023979)	1:161912324-161913262	No homologues	
Olr148 (ENSRNOG00000016993)	1:161948171-161949142	OR52D1 (ENSG00000181609)	11:5509915-5510979
RGD1562433 (ENSRNOG00000036807)	1:161962308-161963840	No homologues	
Ubqln3 (ENSRNOG00000023833)	1:161974585-161977032	UBQLN3 (ENSG00000175520)	11:5528530-5531215
Ubqlnl (ENSRNOG00000017016)	1:161985144-161986982	UBQLNL (ENSG00000175518)	11:5535623-5537935
RGD1310717 (ENSRNOG00000017039)	1:161990223-161998056	No homologues	
Olr149 (ENSRNOG00000017138)	1:162014526-162015476	OR52H1 (ENSG00000181616)	11:5565719-5566779
Olr150 (ENSRNOG00000017143)	1:162032269-162033207	No homologues	
Trim6 (ENSRNOG00000017147)	1:162066157-162072075	TRIM6 (ENSG00000121236)	11:5617339-5634188
D3ZA88_RAT (ENSRNOG00000042686)	1:162086771-162093563	TRIM6-TRIM34 (ENSG00000258588)	11:5617955-5665628
		TRIM34 (ENSG00000258659)	11:5617946-5665628
Trim5 (ENSRNOG00000017191)	1:162096454-162113409	TRIM5 (ENSG00000132256)	11:5684425-5959849
F1LV70_RAT (ENSRNOG00000036804)	1:162142545-162143057	No homologues	
Trim30 (ENSRNOG00000042947)	1:162173307-162174453	No homologues	

Table 3. List of Genes and Genomic Position in the Rat *Mcs3* Locus and in the Orthologous Human Region Obtained Using Ensembl Genome Browser (continued)

Rattus norvegicus		Homo sapiens	
Genes	Location	Homologues	Location
D4A0Y0_RAT (ENSRNOG00000031202)	1:162183971-162184423	No homologues	
D3ZA96_RAT (ENSRNOG00000024179)	1:162195024-162196352	No homologues	
Olr152 (ENSRNOG00000017211)	1:162202138-162203079	No homologues	
Olr153 (ENSRNOG00000017214)	1:162211898-162212872	No homologues	
Olr154 (ENSRNOG00000017218)	1:162225307-162226260	No homologues	
Olr156 (ENSRNOG00000017239)	1:162247587-162248513	No homologues	
Olr157 (ENSRNOG00000017252)	1:162265829-162267222	No homologues	
Olr158 (ENSRNOG00000017261)	1:162286565-162287524	OR56B1 (ENSG00000181023)	11:5757681-5758768
Olr159 (ENSRNOG00000031557)	1:162294975-162295946	No homologues	
Olr160 (ENSRNOG00000031490)	1:162309721-162310695	No homologues	
Olr161 (ENSRNOG00000029963)	1:162319179-162320138	No homologues	
Olr162 (ENSRNOG00000034173)	1:162332962-162333921	No homologues	
D3ZLJ1_RAT (ENSRNOG00000032841)	1:162340898-162342106	No homologues	
Olr163 (ENSRNOG00000031012)	1:162349312-162350277	No homologues	
Usp17l5 (ENSRNOG00000043303)	1:162371236-162372750	USP17L2 (ENSG00000223443)	8:11994677-11996586
		RP11-1396O13.9 (ENSG00000231637)	4:9355364-9356956
		RP11-1396O13.8 (ENSG00000231051)	4:9350619-9352211
		RP11-1396O13.7 (ENSG00000235780)	4:9345874-9347466
		RP11-1396O13.5 (ENSG00000229579)	4:9336384-9337976
		USP17 (ENSG00000232264)	4:9326891-9328483
		RP11-1396O13.4 (ENSG00000230430)	4:9331637-9333229
		RP11-1396O13.11 (ENSG00000228856	4:9364855-9366447
		USP17L5 (ENSG00000227140)	4:9341129-9342721
		RP11-1286E23.15 (ENSG00000250745	4:9259850-9261442
		RP11-1286E23.6 (ENSG00000233136)	4:9217131-9218723
		RP11-1286E23.17 (ENSG00000248933	4:9269345-9270937
		RP11-1286E23.14 (ENSG00000248920	4:9255104-9256696
		RP11-1286E23.13 (ENSG00000250844	4:9250356-9251948
		RP11-1286E23.16 (ENSG00000249811	4:9264598-9266190
		RP11-1286E23.7 (ENSG00000227551)	4:9221878-9223470
		RP11-1286E23.10 (ENSG00000223569	4:9236111-9237700
		RP11-1286E23.8 (ENSG00000232399)	4:9212383-9228214
		RP11-1286E23.12 (ENSG00000249104	4:9245605-9247197
		RP11-1286E23.5 (ENSG00000231396)	4:9212383-9213975
		RP11-1286E23.18 (ENSG00000250913	4:9274090-9274640
Olr164 (ENSRNOG00000033899)	1:162392486-162393451	OR52N1 (ENSG00000181001)	11:5809084-5810046
Olr165 (ENSRNOG00000032268)	1:162420209-162421177	No homologues	
Olr168 (ENSRNOG00000029647)	1:162486804-162487763	No homologues	
Olr170 (ENSRNOG00000029375)	1:162510151-162511116	OR52N1 (ENSG00000181001)	11:5809084-5810046
LOC689730 (ENSRNOG00000017288)	1:162540440-162549650	USP17L2 (ENSG00000223443)	8:11994677-11996586
		RP11-1396O13.9 (ENSG00000231637)	4:9355364-9356956
		RP11-1396O13.8 (ENSG00000231051)	4:9350619-9352211
		RP11-1396O13.7 (ENSG00000235780)	4:9345874-9347466
		RP11-1396O13.5 (ENSG00000229579)	4:9336384-9337976
		USP17 (ENSG00000232264)	4:9326891-9328483
		RP11-1396O13.4 (ENSG00000230430)	4:9331637-9333229
		RP11-1396O13.11 (ENSG00000228856	4:9364855-9366447
		USP17L5 (ENSG00000227140)	4:9341129-9342721
		RP11-1286E23.15 (ENSG00000250745	4:9259850-9261442
		RP11-1286E23.6 (ENSG00000233136)	4:9217131-9218723
		RP11-1286E23.17 (ENSG00000248933	4:9269345-9270937
		RP11-1286E23.14 (ENSG00000248920	4:9255104-9256696
		RP11-1286E23.13 (ENSG00000250844	4:9250356-9251948
		RP11-1286E23.16 (ENSG00000249811	4:9264598-9266190
		RP11-1286E23.7 (ENSG00000227551)	4:9221878-9223470
		RP11-1286E23.10 (ENSG00000223569	4:9236111-9237700
		RP11-1286E23.8 (ENSG00000232399)	4:9212383-9228214
		RP11-1286E23.12 (ENSG00000249104	4:9245605-9247197

Table 3. List of Genes and Genomic Position in the Rat *Mcs3* Locus and in the Orthologous Human Region Obtained Using Ensembl Genome Browser (continued)

Rattus norvegicus		Homo sapiens	
Genes	Location	Homologues	Location
		RP11-1286E23.5 (ENSG00000231396)	4:9212383-9213975
		RP11-1286E23.18 (ENSG00000250913	4:9274090-9274640
Olr171 (ENSRNOG00000031652)	1:162563516-162564466	No homologues	
Olr172 (ENSRNOG00000030695)	1:162571258-162572214	OR52N2 (ENSG00000180988)	11:5841544-5842578
Olr174 (ENSRNOG00000029040)	1:162590020-162590976	OR52N2 (ENSG00000180988)	11:5841544-5842578
Olr175 (ENSRNOG00000017308)	1:162618317-162619282	OR52N2 (ENSG00000180988)	11:5841544-5842578
Olr176 (ENSRNOG00000032615)	1:162626124-162627080	OR52N2 (ENSG00000180988)	11:5841544-5842578
Olr178 (ENSRNOG00000017331)	1:162681198-162682136	No homologues	
Olr179 (ENSRNOG00000033989)	1:162728293-162729231	No homologues	
Olr180 (ENSRNOG00000033813)	1:162739521-162740471	OR52E8 (ENSG00000183269)	11:5877904-5878964
		OR52E6 (ENSG00000205409)	11:5862158-5863182
Olr181 (ENSRNOG00000033534)	1:162772333-162773271	No homologues	
Olr183 (ENSRNOG00000027423)	1:162782725-162783666	No homologues	
Olr184 (ENSRNOG00000017341)	1:162793777-162794730	OR52E8 (ENSG00000183269)	11:5877904-5878964
		OR52E6 (ENSG00000205409)	11:5862158-5863182
Olr185 (ENSRNOG00000017344)	1:162803635-162804588	No homologues	
Olr186 (ENSRNOG00000033218)	1:162810227-162811168	No homologues	
Olr188 (ENSRNOG00000017351)	1:162835732-162836670	OR52E4 (ENSG00000180974)	11:5905501-5906527
Olr189 (ENSRNOG00000031357)	1:162843036-162843977	No homologues	
Olr190 (ENSRNOG00000017357)	1:162857313-162858260	OR56A3 (ENSG00000184478)	11:5968570-5969591
LOC100362148 (ENSRNOG00000017362)	1:162862402-162864364	No homologues	
Olr192 (ENSRNOG00000017366)	1:162881824-162882774	No homologues	
Olr193 (ENSRNOG00000030947)	1:162895667-162896718	No homologues	
Olr194 (ENSRNOG00000029946)	1:162927245-162928195	OR52L1 (ENSG00000183313)	11:6007122-6008215
Olr196 (ENSRNOG00000033931)	1:162966355-162967305	OR52L1 (ENSG00000183313)	11:6007122-6008215
Olr197 (ENSRNOG00000017379)	1:162986552-162987505	No homologues	
Olr198 (ENSRNOG00000032010)	1:163004734-163005699	AC111177.1 (ENSG00000180913)	11:6149840-6150706
Olr199 (ENSRNOG00000029755)	1:163021101-163022051	AC111177.1 (ENSG00000180913)	11:6149840-6150706
Olr200 (ENSRNOG00000029155)	1:163042743-163043708	AC111177.1 (ENSG00000180913)	11:6149840-6150706
Olr201 (ENSRNOG00000017387)	1:163069885-163070844	AC022762.1 (ENSG00000180909)	11:6173006-6173818
Olr202 (ENSRNOG00000025819)	1:163080521-163081489	OR52B2 (ENSG00000255307)	11:6190560-6191638
RGD1561034 (ENSRNOG00000030818)	1:163107431-163110605	C11orf42 (ENSG00000180878)	11:6226796-6232362
Fam160a2 (ENSRNOG00000017408)	1:163110928-163133590	FAM160A2 (ENSG00000051009)	11:6232565-6255941
Cnga4 (ENSRNOG00000017609)	1:163137544-163141556	CNGA4 (ENSG00000132259)	11:6255995-6265659
Cckbr (ENSRNOG00000017679)	1:163156914-163166969	CCKBR (ENSG00000110148)	11:6280966-6293357
Prkcdbp (ENSRNOG00000017914)	1:163212922-163214511	PRKCDBP (ENSG00000170955)	11:6340176-6341877
Smpd1 (ENSRNOG00000017977)	1:163278970-163282812	SMPD1 (ENSG00000166311)	11:6411655-6416228
Apbb1 (ENSRNOG00000018020)	1:163282918-163299333	APBB1 (ENSG00000166313)	11:6416354-6440644
Hpx (ENSRNOG00000018257)	1:163319509-163327017	HPX (ENSG00000110169)	11:6452279-6463847
Trim3 (ENSRNOG00000018356)	1:163331603-163362846	TRIM3 (ENSG00000110171)	11:6469843-6495689
Arfip2 (ENSRNOG00000018440)	1:163364861-163369542	ARFIP2 (ENSG00000132254)	11:6496910-6502666
TIM9B_RAT (ENSRNOG00000018654)	1:163369752-163372576	FXC1 (ENSG00000132286)	11:6502677-6530208
F1M5X6_RAT (ENSRNOG00000043327)	1:163380016-163420661	No homologues	
D4A4C1_RAT (ENSRNOG00000033327)	1:163433572-163436909	DNHD1 (ENSG00000179532)	11:6518490-6593257
Dnhd1 (ENSRNOG00000043240)	1:163462348-163467223	DNHD1 (ENSG00000179532)	11:6518490-6593257
Rrp8 (ENSRNOG00000018766)	1:163477030-163481051	RRP8 (ENSG00000132275)	11:6616305-6624850
Ilk (ENSRNOG00000018993)	1:163481299-163487550	ILK (ENSG00000166333)	11:6624961-6632102
Taf10 (ENSRNOG00000019178)	1:163487518-163488786	TAF10 (ENSG00000166337)	11:6627526-6633898
Tpp1 (ENSRNOG00000019212)	1:163490393-163496526	TPP1 (ENSG00000166340)	11:6634000-6640692
Dchs1 (ENSRNOG00000031643)	1:163497340-163517091	DCHS1 (ENSG00000166341)	11:6642554-6677085
RM17_RAT (ENSRNOG00000019497)	1:163554719-163556345	MRPL17 (ENSG00000158042)	11:6702013-6704632
D4A3E9_RAT (ENSRNOG00000042713)	1:163589107-163590165	No homologues	
RGD1308274 (ENSRNOG00000029048)	1:163590802-163593777	No homologues	
D3ZNN6_RAT (ENSRNOG00000042725)	1:163594662-163595836	No homologues	
D3ZJQ9_RAT (ENSRNOG00000042292)	1:163596557-163598317	No homologues	
D3Z9P7_RAT (ENSRNOG00000042759)	1:163695070-163696128	No homologues	
F1LZ18_RAT (ENSRNOG00000019521)	1:163696150-163696605	No homologues	
F1LV81_RAT (ENSRNOG00000036793)	1:163767761-163768429	ATMIN (ENSG00000166454)	16:81069452-81080963
Olr203 (ENSRNOG00000032322)	1:163776673-163777620	No homologues	
Olr204 (ENSRNOG00000034133)	1:163833782-163834729	No homologues	
Olr205 (ENSRNOG00000029697)	1:163874449-163875399	No homologues	

Position is *Rattus norvegicus* genome build version 3.4

Position is Archive EnsEMBL *Homo sapiens* version 67.37 (GRCh37)

Ensemble genome browser was used to generate table 3. Table 3 lists known genes at the rat *Mcs3* locus and human orthologous loci. Public databases and published literature were searched to identify genes at the *Mcs3* human orthologous loci associated with breast cancer. The cancer portal of Rat Genome Database was used to data mine genes associated with breast cancer. We found three genes within the rat Mcs3 locus and/or the human orthologous loci associated with breast carcinomas. Table 4 lists these genes and known functions. Among these genes, *Pak1, Ilk* and *Il18bp* are common to both humans and rats.

Discussion

The focus of this study was aligned toward providing information regarding the genetic aspect of human breast cancer by using rat mammary carcinoma as a model. By physically confirming and mapping rat *Mcs3*, a previously predicted mammary carcinoma susceptibility locus, we sought to develop genetic strains to better understand the genetic contribution associated with human breast cancer susceptibility, and eventually discover the underlying mechanism associated with breast carcinogenesis. In this study, *Mcs3* was physically confirmed and mapped to a 25.8 Mb region on rat chromosome *1* using congenic lines. The Cop *Mcs3* allele was found to reduce the mammary carcinoma susceptibility phenotype associated with the WF strain by 51% when homozygous. In the previous study, that predicted the *Mcs3* QTL, one Cop allele at the *Mcs3* QTL was predicted to reduce

Table 4. List of Genes Associated with Breast Cancer and Genomic Position in the Rat Mcs3 Locus and in the Orthologous Human Region

Rat Chromosome: base position	Human Chromosome: base position	Human Gene Symbol	Gene Name
1: 155,057,622 - 155,174,714	11: 77,033,060 - 77,185,108	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
1: 159,471,809 - 159,473,288	11: 71,709,955 - 71,713,965	IL18BP	interleukin 18 binding protein
1: 163,481,299 - 163,487,550	11: 6,624,938 - 6,632,105	ILK	integrin linked kinase

Position is *Rattus norvegicus* genome build version 3.4.

Position is *Homo sapiens* genome build version hg19.

This table displays the position of genes within the human and rat genomes. It also shows the Gene symbol and gene name.

mammary carcinoma susceptibility by 42% in female rats that were heterozygous at the *Mcs3* locus [173]. We did not test mammary carcinoma susceptibility in *Mcs3* heterozygous congenic rats. The previous study was performed on F_2 and rats from two successive backcross generations. 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 linkage analysis. In this study, *Mcs3* was mapped to a 25.8 Mb region on rat chromosome *1* between markers *ENSRNOSNP2784088* to *ENSRNOSNP2784322* (~25.8 Mb, 138,096,182 – 163,886,079). This region is distal to the predicted *Mcs3* peak QTL markers *D1Mit11* and *D1Wox6*. This is not the first time that a *Mcs* locus has been physically confirmed outside the predicted QTL interval. Both *Mcs1b* and *Mcs1c* are beyond the predicted *Mcs1* QTL interval [181, 197].

Different segments of the rat *Mcs3* locus aligned to 4 human loci that were on chromosomes *11* and *15,* which are shown in table 2. Altogether, the rat and human regions contained 3 annotated genes that have been shown to associate with breast cancer, namely *serine/threonine-protein kinase PAK 1 (PAK1), integrin-linked protein kinase (ILK) and, interleukin-18-binding protein (IL18BP)*. Serine/threonine-protein kinase PAK 1 (*PAK1*) downstream pathways play an important role in development and maintenance of metastatic phenotypes in breast cancer cells [198]. *PAK1* was also shown to be a predictor of recurrence and tamoxifen resistance in post-menopausal breast cancer [199]. Integrin-linked protein kinase (*ILK*) may be an important marker since increased expression of ILK is associated with aggressive progression and poor prognosis of breast cancer [200]. ILK was shown to regulate γ-secretase-mediated *Notch1* activation in IL-6

induced breast cancer stem cells [201]. Interleukin-18-binding protein (*IL18BP*) regulates the activity of interleukin 18. IL-18 expression is significantly higher in malignant breast tumor tissue as compared to breast tissue of patients with benign breast disease [202]. Another study suggests IL-18 enhances breast cancer cell migration [203].

We conclude that the predicted *Mcs3* locus has been physically confirmed and mapped to a 25.8 Mb region on the rat chromosome *1*. Our analysis suggests that there are at least three potential candidate genes located at the rat and orthologous human *Mcs3* genomic region. The rat *Mcs3* congenic lines developed will be useful tools to identify *Mcs3* candidate genes and study mechanisms underlying breast cancer genetic susceptibility.

Materials and Methods

Congenic Breeding and Genotyping

Congenic lines were generated using methods previously described [175]. Briefly, rats with selected Cop chromosome *1* segments from the predicted *Mcs3* QTL were introgressed onto a WF/NHsd genetic background. Congenic rat lines were maintained in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility on a 12-hour 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 (Louisville, KY). Information for genetic markers defining ends of COP alleles carried by each congenic line A, D, E and G were available at the UCSC Genome Browser (www.genome.ucsc.edu), Rat Genome Database (http://rgd.mcw.edu/), or Table 5. Animals were genotyped as described in [175]. Briefly, tail clips were used for DNA

extraction and genotyping. Five to eight days after birth, the rats were toe tattooed and tail clipped. The DNA was extracted to test the genotype at a given locus. Polymorphic micro-satellite markers that vary between the two strains were selected and were used to PCR amplify the variable region of genomic DNA. The amplified DNA was run on a 3% high resolution agarose gel. After the run was complete, the gel was stained in SYBR Gold and scanned on a Typhoon scanner. Visible bands were analyzed using homozygous Cop and WF, and F1 DNA (heterozygous) as controls. For regions with low density of polymorphic STS markers, informative SNP that were polymorphic between Cop and WF rats were identified using SNPlotyper tool on Rat Genome Database (http://rgd.mcw.edu/). Phenotyped rat DNA were sequenced to identify the genotype. Information for SNP genetic markers defining ends of COP alleles carried by each congenic line A and E used for sequencing are available in Table 5.

Table 5. Primer Sequences for Microsatellites in 25.8 Mb Region of Mcs3 Locus

Table 5. Primer Sequences for Microsatellites and SNV in 25.8 Mb region of Mcs3 locus								
Samuelson Lab ID	Type	Forward Primer Sequence	Reverse Primer Sequence	Position*	Variant Position*	Variant	COP	WF
D1Rat27	STS	GGGCAAGCAAAGTACATGGT	TCTCTCCAGCTGCAGGATTT	90282041-90282193		WF=COP†		
D1Mit30	STS	TGTCTTGGCCTCTGATTTCA	TGCTGTGTGGACGGAGATAA	94473941-94474122		WF=COP†		
D1Rat214	STS	GATTTTCAATCTTTTAGCAAGCA	TTTCAGTGACAACCAGGCTG	100034565-100034664		WF=COP†		
D1Mit11	STS	ATAAGCCAGCCCCCATTC	CCTACTGAAAGTGAAATGTCTGG	102532147-102532417		WF=COP†		
D1Rat32	STS	TGAGCCATTGAGTTGTGAGC	TGTTTTCCAAATGAAGCGTG	112148734-112149024		WF=COP†		
D1Rat381	STS	TCAAAAGGCTAAGGCAGGAA	AGACAAAAATGGAGGCCTCA	115368982-115369185		WF=COP†		
D1Rat320	STS	CTCCACATGCACAGGCATAT	CTCCTAAAGGTCCCATTGCA	119536685-119536913		WF=COP†		
D1Rat321	STS	TGCACCAAATTTTCTATTCCA	ACATTTGCCTTTTTGGACAT	127965057-127965205		WF=COP†		
D1Rat36	STS	TTCCTGGGGTACTCCCAC	TTCCTCTCCTTCAACTCCTCC	128874535-128874670		WF=COP†		
D1Wox6	STS	CCCCATCTATCTATCCAACGG	CTCTGGGATGCTTTGTGAAGG	131956600-131956825		WF=COP†		
D1Rat382	STS	GGCCGAATGCTTTCAATAGA	GGCATACATGCTCAAACTGC	132558148-132558270		WF=COP†		
D1Rat350	STS	CCAGATGAAGGCTGATGGAC	ACACGGTATTCAATCAGCCC	135620323-135620477		WF=COP†		
D1Rat173	STS	GATCCCTTGACAAGCATGGT	GATGGAGGCAGTTTTTCCAA	145283364-145283529		WF=COP†		
D1Mgh8	STS	CCTCTGGATTCTGCCAGAAG	TTTCAAATGTACAGGCTGAAACA	156124625-156124741		WF=COP†		
D1Rat277	STS	TCTGGTCTTTACATGTATGTGCA	TTCACATCAGTTTTGGCCAC	164875097-164875292		WF=COP†		
D1Rat243	STS	TCAACTCCCAGGGATTGTTC	CCCTGCTCTAACCAGCAGTC	172072626-172072864		WF=COP†		
D1Rat65	STS	TGAAGGAGAGCCAGGAATTG	CAGTCTGGGGGTAAGCAAGA	184879920-184880048		WF=COP†		
D1Rat290	STS	GGCTGAGTTTTTCTCTGACACTG	GGTCTCACTTCAGGGAGCAG	195105753-195105954		WF=COP†		
ENSRNOSNP278375	SNP	TTGTCCCTGAGCCTCG	GCCCGTCTCCACTTCT		95200769	C/T	CC	TT
ENSRNOSNP278380	SNP	GGAGTCTGGGCACTGT	ACACCCAGCTAGCAGG		99077826	T/A	TT	AA
ENSRNOSNP278407	SNP	GTGAGCAGCTGTTGGG	GGGAACTCACACCGGA		137304344	T/C	TT	CC
ENSRNOSNP278408	SNP	AGTATCTGCCCGGTGG	GGCAAACCGTCCTGAAA		138096182	G/A	AA	GG
ENSRNOSNP278413	SNP	TGCTTCGCCTTAACCTG	TGCATGTCAGAAGGGAGA		143556969	G/A	GG	AA
ENSRNOSNP278426	SNP	TGACCTCCGTGCTACC	ACCTGGTGTCGCTTCA		158009944	T/A	AA	TT
ENSRNOSNP278432	SNP	AACGGGCCAATGGAATG	CCACCAGCCCCTAAGA		163886079	G/T	GG	TT
ENSRNOSNP278433	SNP	CTGTCACCCCAGCACT	TGATTTGTCCCGGGGA		164656091	T/C	TT	CC

*Position is *Rattus norvegicus* genome build version 3.4

†Microsatellite DNA genotypes were determined using 3% high resolution agarose

Phenotyping

Female congenic and WF/HSd (Harlan) rats were intubated with a single dose of DMBA (65 mg DMBA/kg body mass) in sesame oil at 50-55 days of age. Mammary carcinomas ≥ 3 × 3mm were counted at 15 weeks post-treatment.

Statistical Analysis

Mammary carcinoma multiplicity data were analyzed and congenic rat phenotypes were compared to that of susceptible WF/NHsd controls. Mammary carcinoma multiplicity phenotypes for more than two groups were compared using Kruskal-Wallis nonparametric test. If this test was statistically significant (p value < 0.05), then each group was compared to the WF/NHsd control group by performing multiple Mann-Whitney nonparametric tests. A p value of ≤ 0.05 was considered to be significant.

Comparative Genomics

The human orthologous region and transcripts mapping to the rat Mcs3 locus were identified using the Rat Genome Database, UCSC Genome Browser and the Ensembl Genome Browser. The *Rattus norvegicus* reference genome sequence version 3.4/rn4 and *Homo sapiens* version GRCh37/hg19 were used. The percent of sequence identity between the rat and the human region of interest was identified using the 'Convert' function at the UCSC genome browser.

CHAPTER III

ASSESSING CONGENIC LINE 1B-11 FOR *MCS1B* ASSOCIATED PHENOTYPE

Introduction

This chapter focuses on rat mammary carcinoma susceptibility locus *Mcs1b,* which was identified from the fine mapping of *Mcs1* [181]. *Mcs1* is a quantitative trait locus (QTL) identified by linkage analysis of DMBA sensitive Wistar-Furth (WF) strain and DMBA resistant Copenhagen (Cop) strain [181]. To genetically map the *Mcs1* locus, WF and Cop rats were bred to generate (WF x Cop) F1 rats. F1 rats were bred with WF rats to generate backcross (BC1) rats. BC1 rats were analyzed for linkage between polymorphic microsatellite markers and susceptibility to DMBA induced carcinogenesis, which was measured by tumor number. The *Mcs1* locus was identified on the proximal end of rat chromosome *2*. The Cop allele at the *Mcs1* locus was predicted to reduce DMBA induced rat mammary carcinoma susceptibility [172]. In an extension of this study, BC1 rats were further backcrossed to generate BC2 rats and F1 rats were crossed with each other to generate F2 rats. Linkage analysis, performed in these rats, physically confirmed the locus and also confirmed that the Cop allele at the *Mcs1* locus reduces DMBA induced mammary carcinoma susceptibility [173].

The *Mcs1b* locus was identified by phenotyping *Mcs1* congenic rats with varying lengths of Cop allele across the *Mcs1* locus on a WF genetic background. These rats were given a single dose of DMBA at fifty to fifty five days of age and tumor number was

counted at fifteen weeks post DMBA administration. This led to the identification of the *Mcs1a*, *Mcs1b,* and *Mcs1c* loci. The Cop allele at the Mcs1b locus was identified to reduce DMBA induced mammary carcinoma susceptibility [173]. A recent study by denDekker *et al.* from our lab used *Mcs1b* congenic rats to define this locus to a 1.8 Mb region of rat chromosome 2 [137]. The authors performed mammary gland transplant assays in which mammary glands from the susceptible or resistant rats were transplanted onto the back fat pads of rats with either a susceptible or resistant genotype. These rats were then given a single dose of DMBA and tumor outcome was assessed post DMBA administration. The results of this experiment showed that irrespective of the recipient genotype, the grafts were tumor positive if the donor rat was of the susceptible genotype [137]. Thus, *Mcs1b* acts in a mammary gland autonomous manner. The authors then sequenced the open reading frames of genes present in the *Mcs1b* locus in the WF and Mcs1b congenic rats. No differences were identified in the protein coding region suggesting that *Mcs1b* locus could potentially function by controlling non protein coding regions and differentially regulating gene expression between the two genotypes. Mcs1b gene transcript levels were measured in WF and *Mcs1b* congenic rats. The authors found that *Map3k1*, *Gpbp1*, *Mier3* were differentially expressed between the two genotypes without DMBA exposure, but only *Mier3* was differentially expressed between the two genotypes with DMBA. *Map3k1*, *Gpbp1*, *Mier3* transcript levels were found to be lower for the candidate genes in the Mcs1b resistant rats compared to the susceptible WF rats. [137]. Tumor expression levels of *Map3k1*, *Gpbp1*, *Il6st* and *Mier3* was tested. The authors found that *Mier3* was more highly expressed in tumors as compared to disease free mammary tissue [137]. Lastly, it was also noted that *Mcs1b* female rats had a higher

body weight compared to susceptible WF female rats when weighed at 12 weeks of age [137]. In summary, *Mcs1b* associated phenotypes include reduced tumor number, lower expression levels of *Map3k1*, *Gpbp1*, and *Mier3* in mammary tissue compared to susceptible WF without DMBA exposure, lower expression levels of *Mier3* in mammary tissue compared to susceptible WF with DMBA exposure and higher *Mier3* expression in mammary tumors compared to adjacent disease free mammary tissue.

It is worth noting that *Mcs1b* heterozygous congenic rats tested by Haag *et al*. developed an average of 7.6 tumors/rat [181]; whereas, *Mcs1b* heterozygous congenic rats tested by denDekker *et al*. developed around 5.5 tumors/rat [137]. This discrepancy in number of tumors developed by rats that are heterozygous Cop at the *Mcs1b* locus, observed in two different studies, could be due to the difference in the lengths of the congenic segment contained in the congenic lines tested; and thus, may be indicative of a complex genetic interaction within the *Mcs1b* locus. To test this hypothesis and delimit *Mcs1b* to the narrowest possible interval, it is essential to continue fine mapping the *Mcs1b* locus by phenotyping *Mcs1b* congenic lines with shorter Cop alleles. One such recombinant generated is congenic line 1b-11 which is shown in figure 8. It was generated by backcrossing congenic line T to the parental WF strain. Congenic line 1b-11 has a Cop allele which spans the distal end of the *Mcs1b* locus, which was present in the Mcs1b rats tested by Haag *et al.*, but was only partially present in the *Mcs1b* rats tested by denDekker *et al*. Thus, line 1b-11 is a good candidate to test the hypothesis that the *Mcs1b* locus might interact with a distal locus. To test this possibility, congenic line 1b-11 will be investigated for *Mcs1b* associated phenotypes. Tumor multiplicity post DMBA administration will be compared between congenic 1b-11 and WF rats. *Mier3* expression

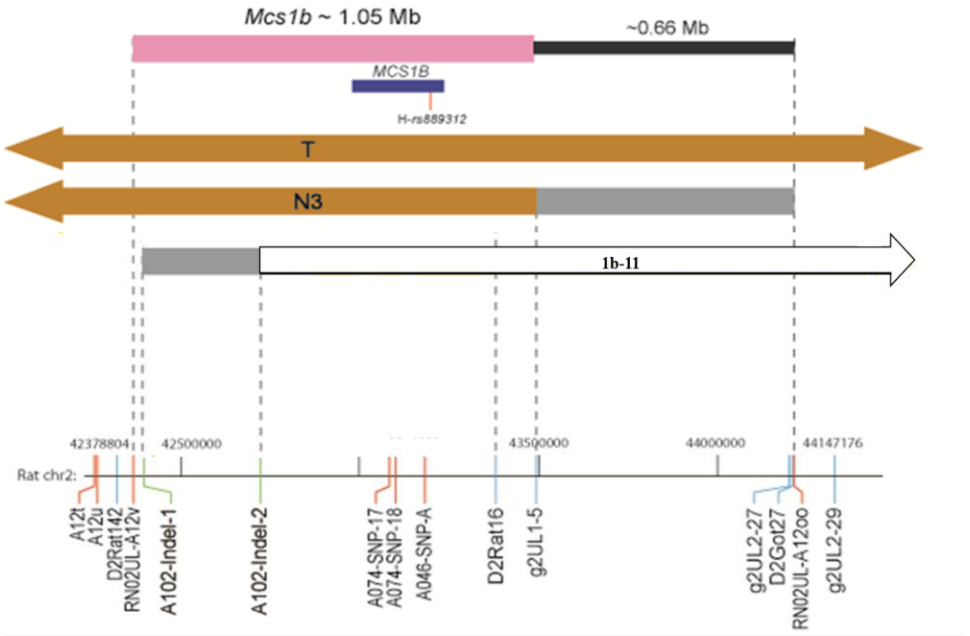


Figure 8. WF.Cop Congenic Map that defines the *Mcs1b* Line 1b-11. Figure represents varying segments on rat chromosome 2 where Mcs1b was predicted to be located and congenic lines that were used to fine-map this locus. On the X axis, blue lines represent informative microsatellite markers, red lines represent informative SNPs, and green lines represent informative indels. Brown bars represent Cop alleles in congenic lines with a resistant phenotype, white bars represent congenic lines with a unknown phenotype, and grey bars represent a potential area of recombination where the genotype isn't determined. The horizontal blue bar represents the orthologous region to human 5q11.2 breast cancer risk (BRCR) associated haplotype block**.**

levels in mammary tumors will be compared to adjacent disease free mammary tissue. *Mier3, Map3k1, Gpbp1* and *Il6st* expression levels in mammary gland will be compared between 1b-11 and WF females at 12 weeks of age with DMBA and without DMBA exposure. Body weights of 1b-11 rats will also be compared to that of WF rats at 12 weeks of age. The goal of this aim was to narrow the Mcs1b QTL interval and potentially identify genetic interactions with another locus.

Results

Analysis of Congenic Line 1b-11 Set Ups

Generating Rats That Were Heterozygous Cop at the 1b-11 Locus

Initially, there was one fertile male rat that was homozygous at the 1b-11 locus. This male was backcrossed with susceptible WF female rats. Table 6 shows that the litters (N17 generation) obtained were heterozygous at the *1b-11* locus. The backcross was successful with eight out of the eleven set ups produced litters (72% of set ups). The litters were comprised of a total of 12 males and 18 females, all of which were heterozygous at the 1b-11 locus. These heterozygous rats were inbred in an attempt to fix this line and obtain female rats that were homozygous Cop at the *1b-11* locus.

Generating Rat That Were Homozygous Cop at the 1b-11 Locus

Rats that were heterozygous at the 1b-11 locus (N17 generation) were set up to generate rats that were homozygous Cop at the *1b-11* locus (Table 6). Out of 41 different set ups only nine (22% of set ups) produced litters (N17F1 generation). There were a total of 22 males and 28 females. N17F1 rats that were homozygous Cop at the *1b-11* locus were set up with rats that were heterozygous Cop at the *1b-11* locus to produce the next generation (N17F2). Fourteen out of thirty five set ups (40% of set ups) generated litter.

Table 6. Analysis of Congenic Lines 1b-11 and *Mcs3* Line D Set Ups

generation	Mcs locus	congenic line	Breeding Pairs	Litters	Male pups	Female pups	% Set ups that produced litters
N17	Mcs1b	1b-11	11	8	12	18	72.73
N17F1	Mcs1b	1b-11	41	9	22	28	21.95
N17F2	Mcs1b	1b-11	35	14	16	25	40.00
N17F3	Mcs1b	1b-11	32	3			9.38
N10F4	Mcs3	Line D	10	5	14	17	50.00
N10F5	Mcs3	Line D	8	2	3	3	25.00
N10F6	Mcs3	Line D	9	4	5	5	44.44
N10F7	Mcs3	Line D	11	5	10	10	45.45

Table provides the number of Mcs1b line 1b-11 and Mcs3 line D breeding pairs set up and the number of set ups that generated litters. It is further broken down to the number of males and females that the litters together contained. The last column is the percentage of set ups that resulted in litters.

There were a total of 16 males and 25 females. To generate female rats that were homozygous Cop at the *1b-11* locus to use in designed experiments male rats and female rats that were homozygous Cop at the *1b-11* locus (N17F3 generation). In this case only three of the thirty-two setups generated litters (9% of set ups). At this point, a decision was made to stop breeding congenic line *1b-11* since it was not generating enough litters.

Analysis of *Mcs3* Congenic Line D Set Ups

Generating Rats That Were Homozygous Cop at the Mcs3 Line D Locus

Rats that were homozygous Cop at the *Mcs3* line D locus (N10F3 generation) were set up to generate the N10F4 generation (Table 6). Out of 10 different set ups, five (50% of set ups) produced litters. There were a total of 14 males and 17 females. Since all the rats that were set up were homozygous Cop at the *Mcs3* Line D locus all *Mcs3* line D litters generated were also homozygous Cop at the *Mcs3* Line D locus. N10F4 rats were set up with each other to produce the next generation (N10F5). Two out of eight set ups (25% of set ups) generated litter. There were a total of 3 males and 3 females. N10F5 rats were set up to produce the next generation (N10F6). Four out of nine set ups (44% of set ups) generated litter. There were a total of 5 males and 5 females. Like the previous three generations the N10F6 generation also bred successfully to generate litters. N10F6 rats were set up with each other to produce the next generation (N10F7). Five out of eleven set ups (45% of set ups) generated litter. There were a total of 10 males and 10 females.

Discussion

The goal of this specific aim was to narrow the *Mcs1b* locus and identify possible complex genetic interactions within the locus. To achieve this goal; congenic line 1b-11

rats were needed. However, congenic line 1b-11 did not breed successfully. *Mcs3* congenic line D breeding is also analyzed to display that congenic line 1b-11 was breeding poorly. In comparison to congenic line 1b-11, congenic *Mcs3* line D always had more than 20% of set ups generate litters with larger litter sizes. Congenic line 1b-11 rats bred successfully when they were heterozygous Cop at the *1b-11* locus. However, most breeders failed to generate litters when rats that were homozygous Cop at the *1b-11* locus were bred with each other. In the event that set ups resulted in a litter, the litter either died at birth or the female ate her litter soon after. The three litters that did survive had a small litter size of 2 to 3 pups each. Thus, it was not feasible to generate litters that were homozygous Cop at the 1b-11 locus.

In general, congenic rats which breed successfully produce a new generation after four months. Congenic line 1b-11 rats were slower to breed since fewer rats were fertile and took approximately five to six months between generations. The breeding issues associated with congenic line 1b-11 rats were noted after eighteen to twenty four months of breeding (four generations of breeding). It was still possible to use rats that were heterozygous Cop at the *1b-11* locus instead of rats that were homozygous Cop at the *1b-11* locus to determine if congenic line 1b-11 replicated *Mcs1b* associated phenotypes. However, a larger sample size of these heterozygous rats would be needed to study the same phenotypes. Due to the above noted difficulties in breeding congenic line 1b-11 and time restrains, the decision was made based on advice from my dissertation committee to not continue with this specific aim.

There are many factors that affect breeding of congenic rats, some of them include first mating age, light intensity, stress, pheromones and diet [204-207]. The

microenvironment comprises the immediate physical enclosure the animal is in. It includes the cage, bedding, food and water supply. The microenvironment is also characterized by illumination, noise, vibration, humidity, temperature and the air within the cage. The macroenvironment comprises the physical environment of the secondary enclosure that houses the primary enclosure. This is the room that contains the rat housing cages. The macroenvironment is affected by the design of the animal room, the traffic within of animal handlers, animal cages, noise, etc. Although the two environments are associated with each other, there are differences between the two. The temperature, humidity and illumination vary appreciably between the macro and microenvironments. Identifying the causes that resulted in congenic line 1b-11 not producing ample litters is beyond the scope of this project. Future research could be directed toward addressing this problem.

Materials and Methods

Generating WF.Cop Congenic Line 1b-11

All animals used for this study were housed within the University of Louisville Research Resources Center Animal Facility. All animal protocols were approved by the University of Louisville IACUC.

Line 1b-11 was developed through a backcross of *Mcs1b* resistant WF.Cop line T to WF rats. The resultant pups were genotyped using informative markers. The recombinant rat with a Cop allele represented in the congenic line 1b-11 was selected and further backcrossed to WF females to expand the line. Rats that are heterozygous Cop at the 1b-11 locus were used in brother sister matings to inbreed the line and generate rats that were homozygous Cop at the 1b-11 locus.

Genotyping

Pups were tail clipped at five to ten days of age and toe tattooed for within litter identification. Genomic DNA was extracted to determine genotypes as described in chapter 2. SNPs between the two strains were used for genotyping. DNA was amplified using TaqMan genotyping master mix from Life Technologies and primers and probes specific for SNP markers. Analysis was performed on StepOne Plus QPCR machine ABI using the StepOne software for genotyping Analysis. Informative markers used to genotype congenic line 1b-11 are shown in table 7.

Table 7. Informative Genetic Markers Used to Genotype Congenic Line 1b-11

Primer and probe Sequences for SNPs in 1b-11 locus					
Samuelson Lab ID	**Type**	**Forward Primer Sequence**	**Reverse Primer Sequence**	**Probe-Copenhagen allele**	**Probe-Wistar-Furth allele**
A12v Allelic Discrimination Assay	SNP	CAACTCATGTTCAGAGACATTTTCCT	TGTGTGGTCTTCTGTTTTCTTGGA	VIC-AGACTTCTCAAACCAG-MGBNFQ	6FAM-AGACTTCTCCAACCAG-MGBNFQ
A12oo Allelic Discrimination Assay	SNP	AACGTTACCGCATTGACACTTG	CAGGCTAGCCTGGGCTATAAGAA	VIC-AAGATTTTTATTTGTTTCAACCAG-MGBNFQ	6FAM-AAGATTTTTATTTGTTTGAACCAG-MGBNFQ
A46-SNP-A Allelic Discrimination Assay	SNP	GCACTCCAGTGGTAAGACTAAAGCA	TTTACCTGGAAACGGAATGCA	VIC-AGAACTTCTACAGAACCT- MGBNFQ	6FAM-AGAACTTCTACAGGACCT-MGBNFQ
A74-SNP-17 Allelic Discrimination Assay	SNP	CTGGGTCAACAACTCGCTGAT	GGAACGTAAGAAACGCTGTAGAGAT	VIC- AGCTGCAAATGTC-MGBNFQ	6FAM-AGCTGCACATGTCAT-MGBNFQ

CHAPTER IV

MCS1B CANDIDATE GENE EXPRESSION IN RAT MAMMARY EPITHELIAL CELLS

Introduction

This chapter focuses on the investigation of mammary epithelial cell populations (MECs) contained in congenic *Mcs1b* rat mammary glands. The rat *Mcs1b* locus was mapped to a 1.8Mb region on rat chromosome *2* using congenic rats with *Mcs1b* Copenhagen (Cop) alleles on a Wistar-Furth (WF) genetic background by denDekker *et al* [137]. The Cop allele at the *Mcs1b* locus confers resistance to DMBA induced mammary carcinogenesis. The *Mcs1b* locus acts in a mammary gland independent manner [137]. Mammary glands from susceptible or *Mcs1b* resistant donor genotypes were transplanted into the interscapular fat pads of susceptible or *Mcs1b* resistant recipient rats. The recipient rats were then given a single dose of DMBA at fifty to fifty five days of age. Tumor outcome was checked at fifteen week post DMBA administration on the transplanted mammary gland. The donor genotype, but not the recipient genotype had a significant effect on tumor outcome, suggesting a mammary gland independent mechanism for the *Mcs1b* locus. The authors also proceeded to look for sequence variation in open reading frames of *Mcs1b* encoded genes between susceptible and *Mcs1b* resistant rats in an attempt to identify causative genes. No variation was identified in the coding regions of genes in the *Mcs1b* locus. With this observation, it was hypothesized that the *Mcs1b* variant is present in a non-coding region and is responsible for differential

expression of *Mcs1b* encoded genes. Since the *Mcs1b* locus acts in a mammary gland independent manner, the expression assays were performed on mammary tissue extracts. *Mitogen-Activated Protein Kinase Kinase Kinase 1* (*Map3k1*), *GC-Rich Promoter Binding Protein 1* (*Gpbp1*) and *Mesoderm Induction Early Response 1, Family Member 3* (*Mier3*) were differentially expressed between the susceptible and *Mcs1b* resistant mammary glands that were not exposed to DMBA. However, only *Mier3* was differentially expressed between susceptible and *Mcs1b* resistant mammary glands with DMBA exposure. Mammary transcript levels were lower in the *Mcs1b* resistant mammary glands compared to the susceptible mammary glands for all three genes. *Mier3* had a higher expression in tumors as compared to adjacent disease free tissue [137]. A *Mier3* variant (*Mier3-alt5'P*) which uses an alternative 5'start site was also identified. This variant had a higher expression in susceptible mammary glands as compared to *Mcs1b* resistant mammary gland. Since there are two variants of *Mier3* being quantified in the rat mammary gland the variant previously referred to as *Mier3* will be referred to as the full-length variant of *Mier3* and the variant with the alternate 5' start site will be referred to as *Mier3-alt5P*.

The next step of the above investigation was directed toward localizing *Mier3* expression in the mammary gland. The rat mammary gland is a complex tissue. It undergoes most of its development after birth in phases which run parallel to sexual development and reproduction. The rudimentary mammary gland present at birth undergoes development once puberty begins as a result of changes in sex hormone levels. Structurally, the rat mammary gland is a tree like structure, consisting of a network of ducts and lobules. The mammary gland develops ductal structures which consist of

terminal end buds (TEB). These structures grow and branch throughout the mammary gland. The TEBs consist of a hollow central lumen which is lined by layers of cells. The lumen is surrounded by a layer of luminal epithelial cells which are surrounded by a layer of basal epithelial or myoepithelial cells which are further lined by a basement membrane. The tip of the growing end of TEBs consist of cap cells, which are loosely adhering epithelial cells that lack cytoplasmic polarity, steroid receptors, and an organized cytoskeleton [208]. Apart from the ductal structures, the mammary gland comprises the mammary fat pad which contains fibroblasts, endothelial cells, leukocytes and adipocytes [209]. *MIER3* is an uncharacterized gene with a protein product localized to the nucleus [137]. To visualize *Mier3,* Dr. Xu performed immunohistochemistry staining for *Mier3* in *Mcs1b* rat mammary gland tissue. He identified that *Mier3* is not uniformly expressed throughout mammary gland tissue, but is specific to a sub-population of epithelial cells lining the duct (figure 9). Also, as mentioned above, the *Mier3* variant (Mier3-alt5'P) with an alternative 5' transcriptional start site is differentially expressed between *Mcs1b* resistant and susceptible mammary glands. Differentially expressed 5' start variants are typically indicative of cell or tissue type specific expression. It is hypothesized that differential expression of *Mier3* in rat mammary epithelial cells (MECs) explains the reduced susceptibility to DMBA induced mammary carcinogenesis in resistant Mcs1b rats compared to susceptible WF rats. To test this hypothesis, *Map3k1*, *Gpbp1*, full-length *Mier3* and *Mier3-alt5'P* transcript levels were compared between mammary epithelial cell enriched extracts (MEC extracts) obtained from *Mcs1b* resistant and susceptible mammary glands with and without DMBA

exposure. In addition, the MEC extracts were also characterized by quantifying the number of luminal and basal epithelial cells present.

Results

Immunofluorescence of Rat Mammary Gland Was Not Specific

The rat cervical mammary gland sections were stained for Mier3 (stained red in images) and epithelial cells, which were visualized by staining with FITC conjugated

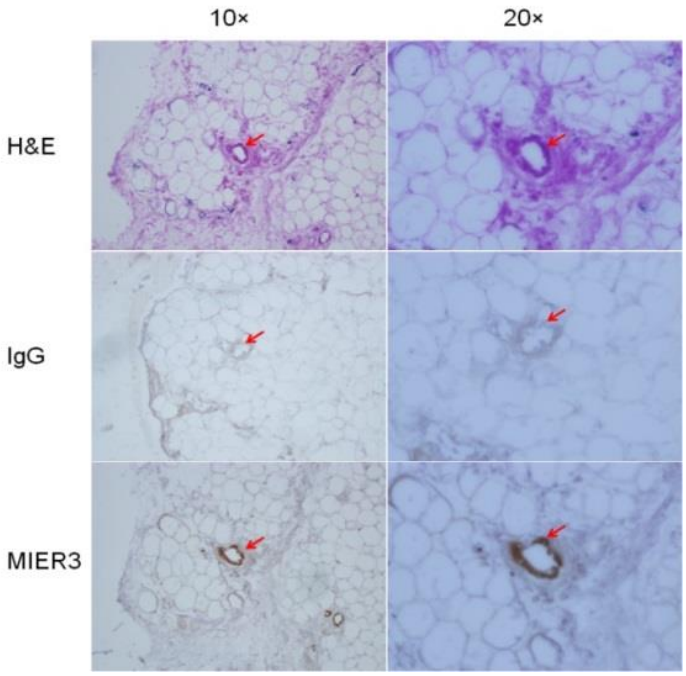


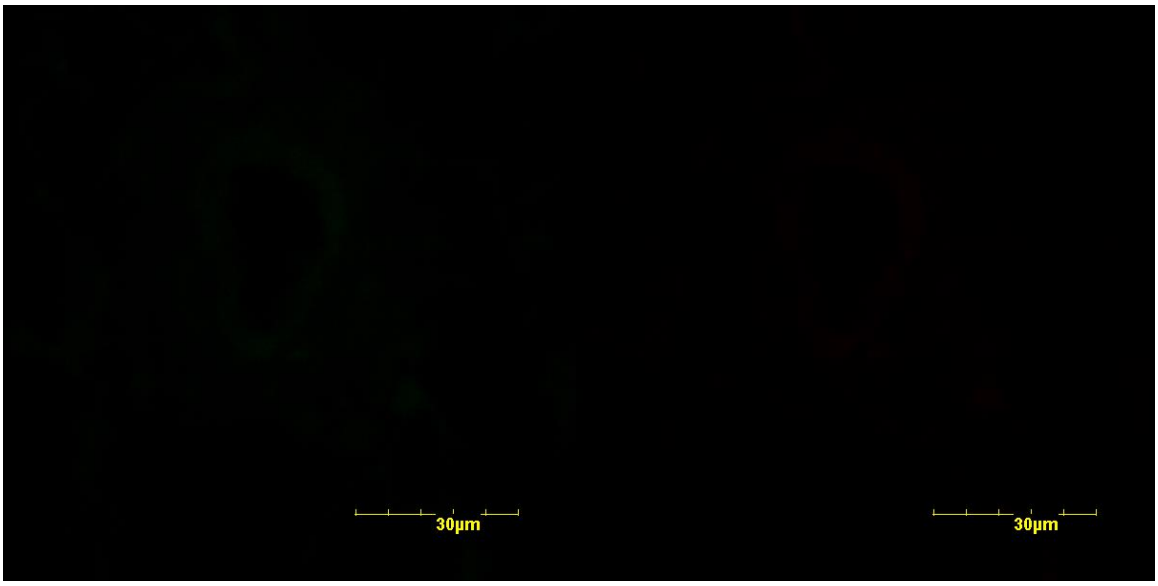
Figure 9. Rat Mammary Gland Stained by Immunohistochemistry for *Mier3*. The above tissues were stained by Dr. Xu. Cervical mammary glands were sectioned and stained for *Mier3* by immunohistochemistry. The first panel shows an H&E stained section of the *Mcs1b* rat mammary gland under 10x and 20x magnification. The second panel shows a control section stained for IgG antibody under 10x and 20x magnification. The third panel shows a mammary tissue section stained for Mier3 antibody under 10x and 20x magnification. *Mier3* appears to be expressed in cells surrounding the duct compared to the rest of the tissue section.

peanut lectin (PNL) antibody (stained green in images). The microscope settings used to visualize the tissue did not capture any tissue auto fluoresce as shown in figure 10A. The Mier3 antibody used binds to Mier3 successfully as shown in figure 10B. However, the Mier3 secondary antibody by itself appeared to bind tissue nonspecifically when used without the Mier3 primary-antibody, as seen in figure 10C. The FITC conjugated PNL antibody by itself also appeared to bind the tissue nonspecifically as well, as seen in figure 10D. This non specificity although minimal in both cases (Figure 10C and figure 10D) was amplified to a much greater extent when the Mier3 secondary antibody was used in conjunction with FITC conjugated PNL antibody, as represented in figure 10E. Thus, although the Mier3 antibody appears to stain MECs in figure 10E, we cannot be certain that the Mier3 secondary antibody and FITC conjugated PNL antibody staining was specific.

Quantification of Luminal Epithelial and Basal Epithelial Populations within MEC Extracts

The isolation of single cell suspensions enriched for MECs was optimized from the protocol found in [210]. Aliquots of MEC extracts were analyzed by flow cytometry with the aim of quantifying luminal and basal cell populations in *Mcs1b* and susceptible WF rats with DMBA exposure and without. Single cells were identified from the population based on forward and side scatter widths. Dead cells were gated out of the single cell populations by using a viability stain. Cells that were CD45+ and CD31+ were gated out, as these were hematopoietic and endothelial cells, respectively. Next, the selected cell population was separated into basal and luminal cell populations, based on CD29 and

A



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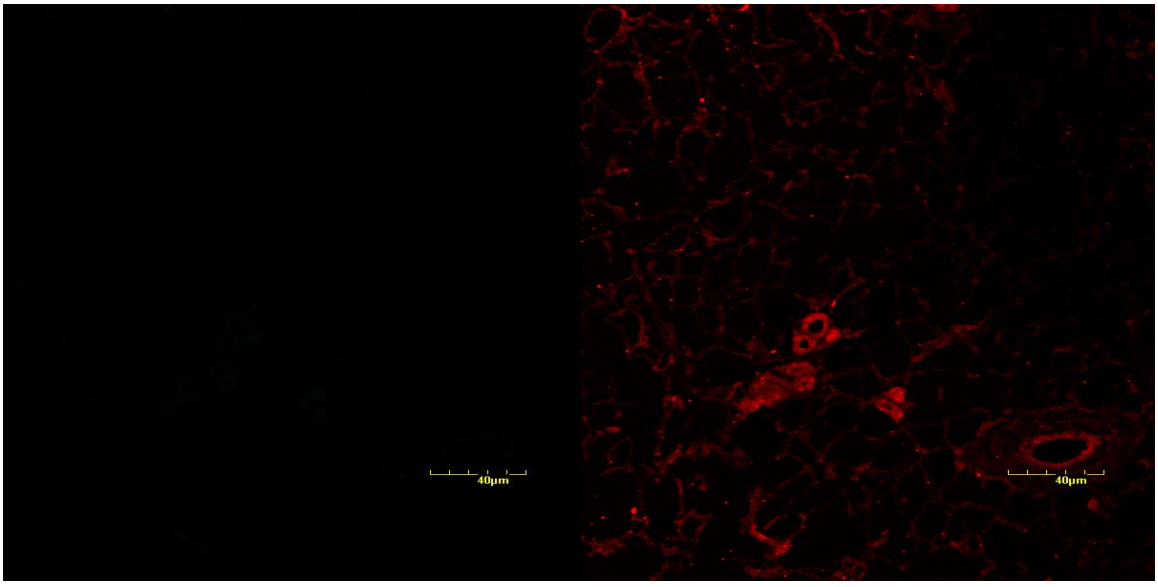

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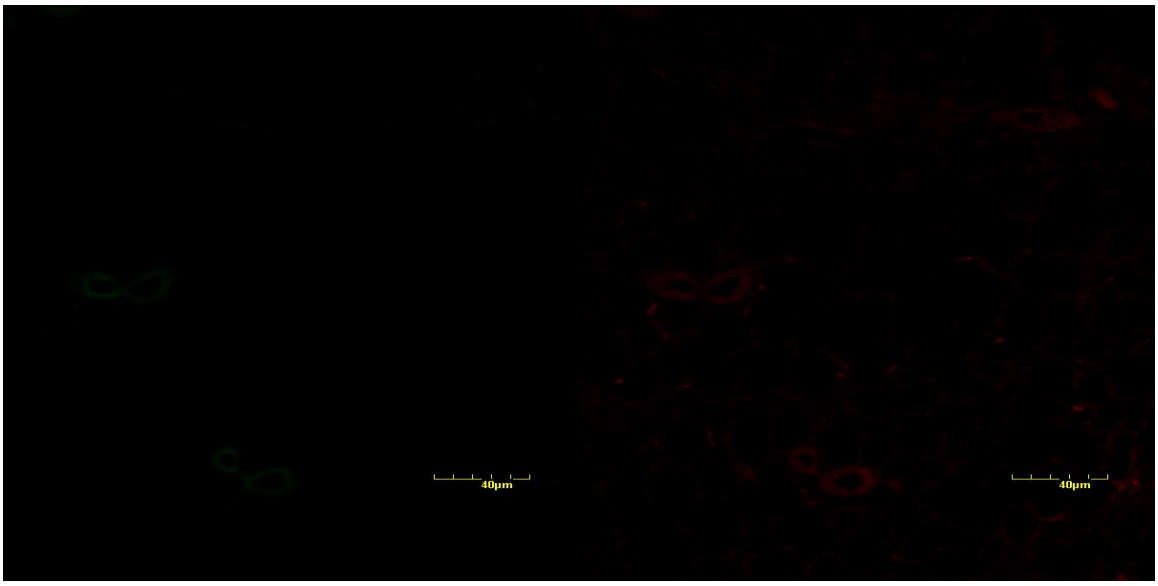

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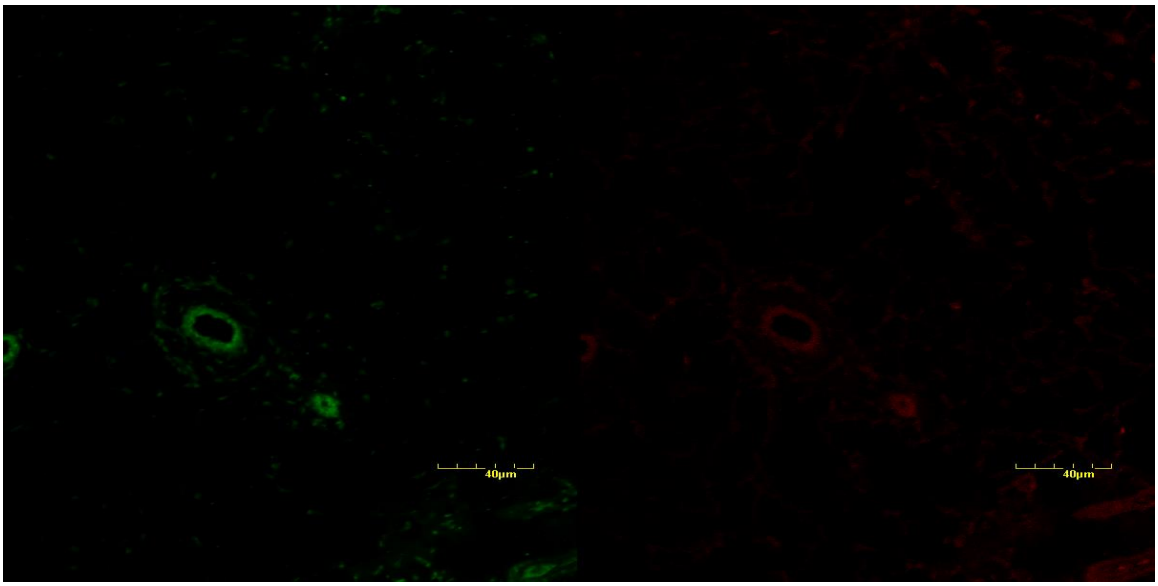

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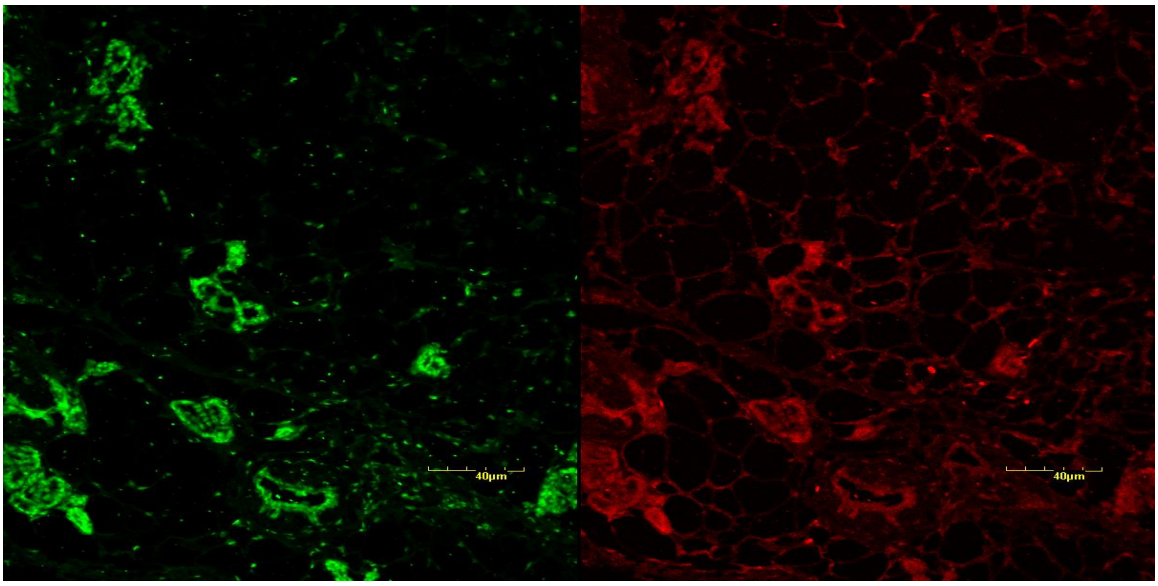

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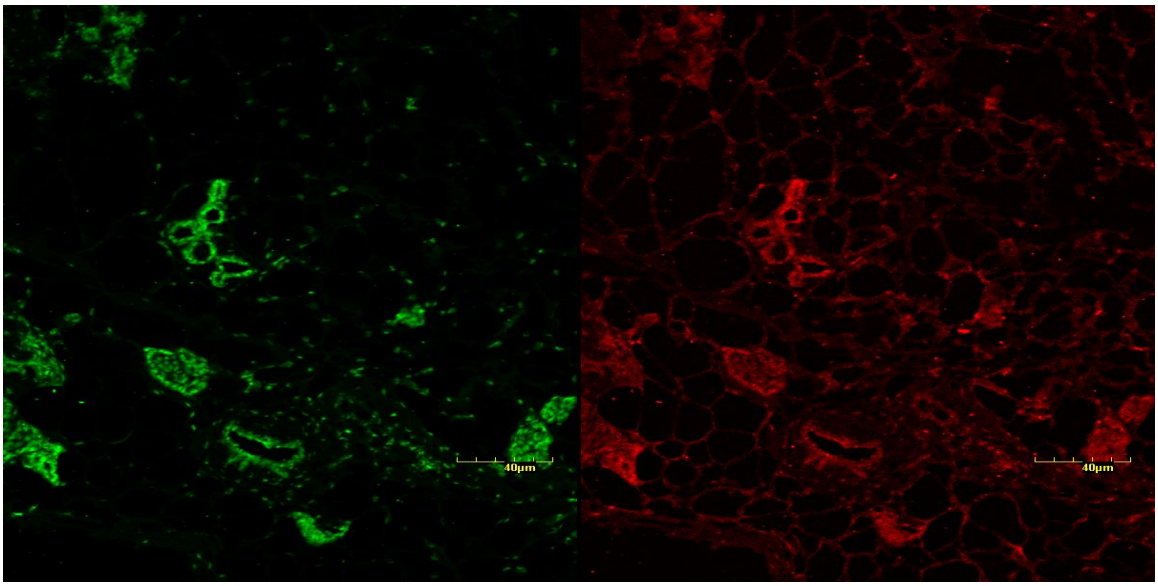

Figure 10. Rat Mammary Gland Immunofluorescence Images. Rat cervical mammary gland sections were stained for *Mier3* (stained red in images) and epithelial cells were visualized by staining with FITC conjugated peanut lectin (PNL) antibody (stained green in images). B to E show mammary gland tissues stained by different combinations of antibodies. A is a picture of the negative control where the mammary gland is not stained with any antibody and it shows no fluorescence. B is a picture of mammary gland tissue stained by *Mier3* primary and secondary antibody. C is a picture of mammary gland stained only by *Mier3* secondary antibody. There appears to be some non-specific binding which is picked up by the red filter. D is a picture of mammary gland stained only by FITC conjugated PNL antibody. There appears to be some non-specific binding which is picked up by the green filter. E represents the rat mammary gland stained for mammary epithelial cells by FITC conjugated PNL and *Mier3* secondary antibody. The non-specificity of the *Mier3* secondary antibody seems to be amplified by the FITC conjugated PNL. F shows mammary gland stained by FITC conjugated PNL and *Mier3* primary and secondary antibodies.

CD24 expression levels. CD24+CD29hi are the basal cell population while CD24+CD29med are the luminal cell population.

The effects of genotype and DMBA exposure on the differences in expression levels were analyzed by performing a two-way ANOVA. This was followed by *post hoc* t-tests. The results are summarized in table 8. Basal cell numbers were not different between *Mcs1b* resistant and susceptible MEC extracts with DMBA exposure (p=0.7996) or without DMBA exposure (p=0.7866). The effect of *Mcs1b* genotype was statistically significant (p=0.0002) for the luminal cell population. There was also a significant effect (p=0.0441) of DMBA exposure on the luminal cell population. When *Mcs1b* genotypes were compared by exposure, luminal cell population levels were significantly different with DMBA exposure (p=0.0193) or without DMBA exposure (0.0009). To summarize, luminal cell numbers were higher in MEC extracts of *Mcs1b* resistant females as compared to susceptible WF females.

Table 8. Analysis of Luminal and Basal Cells from MEC Extracts Obtained at 12 Weeks of Age from *Mcs1b* Congenic and WF Rat Mammary Glands

	Two way ANOVA p-value				percent of parent population average ± SE (n)		
Target	Mcs1b Genotype	Exposure	G x E	Exposure	Susceptible	Mcs1b resistant	t-Test p-value
Luminal MEC	0.0002	0.0441	0.4559	Control	8.68 ± 0.78 (8)	15.94 ± 1.68 (8)	0.0009
				DMBA	11.11 ± 1.46 (7)	23.48 ± 4.62 (8)	0.0193
Basal MEC	0.9514	0.3202	0.7142	Control	22.95 ± 2.25 (8)	24.35 ± 3.25 (8)	0.7996
				DMBA	26.47 ± 1.84 (7)	25.89 ± 2.80 (8)	0.7866
ratio of number of cells to the parent population were square root transformed for analysis purposes							

MEC extracts were analyzed by flow cytometry. Endothelial and hematopoietic cells and dead cells were excluded from the MEC extract to generate the parent extract. The number of luminal MECs and basal MECs were calculated as a percent of the parent extract. Luminal and basal MECs were analyzed by performing a two way ANOVA using Mcs1b genotype (G) and DMBA exposure (E) as independent variables. MEC cell numbers were then compared between the genotypes under different conditions of DMBA exposure by performing a t-Test.

Gene Expression Analysis in MEC Populations Supports *Map3k1, Gpbp1* and *Mier3-Alt5P* as *Mcs1b* Candidate Genes

Previous studies from our lab discovered that full-length *Mier3, Gpbp1, Map3k1* and *Mier3-Alt5P* were differentially expressed between susceptible WF and resistant *Mcs1b* congenic mammary glands without DMBA exposure. However, only full-length *Mier3* was differentially expressed between WF and *Mcs1b* congenic mammary glands with DMBA exposure. Studies from our lab also identified that *Mier3-Alt5P* transcript was differentially expressed between WF and *Mcs1b* mammary tissue, and this difference was not observed between spleen, ovarian or thymus tissues. Also, immunohistochemically stained mammary glands showed that full-length *Mier3* was more highly expressed in cells surrounding the mammary duct compared to surrounding mammary tissue. This led to the hypothesis that *Mcs1b* MECs differentially expressed full-length *Mier3, Map3k1, Gpbp1* and *Mier3-Alt5P* compared to WF MECs. To test this hypothesis, I performed qPCR for these genes in MEC extracts obtained from 12 week old rats that were administered DMBA at 50-55 days of age. *Mcs1b* rats were compared to susceptible WF rats. These rats were also grouped based on DMBA exposure.

The effects of genotype and DMBA exposure on the differences in expression levels were analyzed by performing a two-way ANOVA and this was followed by *post hoc* t-tests. The results are displayed in table 9. Two Mier3 probes were used to test for two of the isoforms of *Mier3* expressed in the mammary gland. The probe referred to as *Mier3* amplified the full-length variant quantified by denDekker *et al* whereas, *Mier3-alt5P* referred to the variant with an alternative 5' start site [137]. The effect of *Mcs1b* genotype was statistically significant ($P < 0.05$) for

Table 9. Analysis of *Mcs1b* Candidate Genes from MEC Extract Obtained at 12 Weeks of Age from *Mcs1b* Congenic and WF Rat Mammary Glands

	Two way ANOVA p-value				Fold change, target vs Rplp2 ± SE (n)		
Target	Mcs1b Genotype	Exposure	G x E	Exposure	Susceptible	Mcs1b resistant	t-Test p-value
Map3k1	0.0001	0.0452	0.7883	Control	1.353 ± 0.138 (12)	0.871 ± 0.123 (19)	0.0170
				DMBA	1.848 ± 0.274 (12)	1.000 ± 0.111 (14)	0.0058
GpBp1	0.0477	0.1199	0.0041	Control	1.257 ± 0.111 (12)	0.842 ± 0.052 (19)	0.0007
				DMBA	1.074 ± 0.108 (12)	1.192 ± 0.088 (14)	0.4003
Mier3	0.7230	0.6785	0.1500	Control	0.932 ± 0.124 (12)	1.216 ± 0.164 (19)	0.2250
				DMBA	1.274 ± 0.195 (12)	1.086 ± 0.130 (14)	0.4177
Mier3-Alt5P	0.0004	0.3538	0.2987	Control	1.481 ± 0.151 (12)	0.994 ± 0.150 (19)	0.0381
				DMBA	1.875 ± 0.311 (12)	0.914 ± 0.126 (14)	0.0059

Mier3 refers to the full length variant of *Mier3*

MEC extracts were aliquoted for gene expression analysis. Expression was calculated by performing QPCR. Differences in expression levels were analyzed by performing a two way ANOVA using Mcs1b genotype (G) and DMBA exposure (E) as independent variables. Expression for every gene was then compared between the genotypes under different conditions of DMBA exposure by performing a t-Test.

Map3k1 (p=0.0001), *Gpbp1* (p=0.0477) and *Mier3-Alt5P* (p=0.0004). Full-length *Mier3* transcript levels were not different between *Mcs1b* resistant and susceptible MEC extracts with DMBA exposure (p=0.2250) or without DMBA exposure (p=0.4177). There was a significant effect of DMBA exposure in *Map3k1* levels (p=0.0452). There was a significant interaction effect between *Mcs1b* genotype and DMBA exposure for *Gpbp1* (p=0.0041). When *Mcs1b* genotypes were compared by exposure, *Map3k1* (p=0.0170), *Gpbp1* (p=0.0007) and *Mier3-Alt5P* (p=0.0381) transcript levels were different between genotypes in groups that were not exposed to DMBA, and only *Map3k1* (p=0.0058) and *Mier3-Alt5P* (p=0.0059) were different between genotypes in groups that were exposed to DMBA. *Map3k1* and *Mier3-Alt5P* transcript levels were lower in resistant *Mcs1b* MEC extracts compared to susceptible WF MEC extracts.

Discussion

Immunofluorescent Staining of *Mier3* and MECs in Rat Mammary Gland Tissue Sections

Mammary gland tissues sections were stained with *Mier3* antibody and FITC conjugated PNL antibody to visualize *Mier3* protein and MECs, respectively. Selected filters did not reveal that any of the tissues auto-fluoresced. Further, the *Mier3* antibody used indicated successful display of sites of *Mier3* expression; it seemed the *Mier3* secondary and FITC conjugated PNL antibodies used were not specific. The *Mier3* secondary antibody stained cells even when the primary antibody was not present. The *Mier3* secondary antibody staining was not as strong without the use of the primary antibody. However, once FITC conjugated PNL was used in combination with the *Mier3* secondary antibody, the non-specificity associated with the *Mier3* secondary antibody

and FITC conjugated PNL antibody was amplified. It appears like the secondary *Mier3* antibody interacted with FITC conjugated PNL. Thus, there is non-specificity associated with the *Mier3* secondary antibody and FITC conjugated PNL. The experiment was not continued due to the lack of time needed to troubleshoot this experiment. Future experiments designed to identify cellular localization of *Mier3* gene products would need to address this issue and identify antibodies that are specific to *Mier3* and MECs individually and do not cross-react.

Characterization of MEC Extract

The goal of this aim was to characterize the MEC extract to support work toward identifying the mechanism of reduced mammary cancer susceptibility associated with *Mcs1b*. Two aspects of MEC extract were quantified. The number of luminal and basal mammary epithelial cells, and transcript levels of full-length *Mier3*, *Gpbp1*, *Map3k1* and *Mier3-Alt5P* present in the MEC extract. The number of luminal and basal cells and full-length *Mier3*, *Gpbp1*, *Map3k1* and *Mier3-Alt5P* expression levels were quantified in MEC extracts obtained from *Mcs1b* resistant and susceptible mammary glands with or without DMBA exposure.

Since the year 2000, breast cancer has also been classified into molecular subtypes based on global gene expression profiles. These five categories, namely luminal A, luminal B, Her2-enriched, basal like and normal breast like group [211]are found across all breast cancer stages from *in situ* carcinomas, inflammatory tumors, and metastatic tumors [212]. Luminal A tumors account for about 50% of invasive breast cancer. Luminal A tumors are estrogen receptor (ER)+, progesterone receptor (PR)+ and Her2-. They are less proliferative, poorly sensitive to chemotherapy, but are responsive to

hormone therapy. Luminal B tumors are ER+, PR+ and may be Her2+ or Her2-. Luminal B tumors have low expression of hormone receptors and they account for 20% of invasive breast cancers. Tumors that overexpress Her2/neu are usually ER-, PR- and have high expression of Her2/neu receptors. These tumors mostly have lymph node metastasis, are of a high grade, and account for 15% of invasive breast cancers. Basal like mammary tumors are similar to basal epithelial and normal myoepithelial cells. It is usually ER-, PR-, Her2/neu- and are CK5/6+ or EGFR+. They account for 15% of aggressive breast cancers. Basal like tumors are mostly high grade invasive cancers and special subtypes of intraductal carcinomas with good prognosis. Luminal B, Her2-enriched and basal like are generally resistant to hormone therapy but sensitive to chemotherapy [213]. In summary, mammary tumor subtypes are associated with tumor prognosis and therapeutic responses.

My studies indicate that *Mcs1b* resistant mammary glands contain a larger population of luminal cells with or without DMBA exposure as compared to *Mcs1b* susceptible mammary glands. Also, exposure to DMBA results in an increased number of luminal epithelial cells in both *Mcs1b* resistant and susceptible WF mammary glands. This indicates that because resistant rats have a larger proportion of luminal epithelial cells with DMBA and without that more MECs might survive the DNA-damaging effects of DMBA or the resistant genotype might be better at replacing MECs. Never the less, results from my experiments suggest that luminal epithelial cell populations are a potential target of DMBA carcinogenesis and functional differences in this population may confer genetically determined differences in susceptibility. Future experiments should be directed towards closely looking at this population for potential expression level differences and effects on carcinogenesis.

I also found that *Map3k1*, *Gpbp1* and *Mier3-Alt5P* were differentially expressed between *Mcs1b* resistant and susceptible mammary glands that had not been exposed to DMBA. However, only *Map3k1* and *Mier3-Alt5P* were differentially expressed between susceptible and resistant MEC extracts after DMBA exposure. The interaction between genotype and DMBA exposure was significant for *Gpbp1* expression, but *Gpbp1* was not differentially expressed between *Mcs1b* resistant and susceptible mammary glands that were exposed to DMBA. Thus, *Map3k1*, *Gpbp1* and *Mier3* are likely candidate susceptibility genes.

MAP3K1 is a serine/threonine kinase which was identified in 1994 [214]. It is known to be a part of ERK and JNK kinase pathways and the NF-kappa-B pathways. Breast cancer susceptibility SNP *rs889312* marks a breast cancer risk associated locus identified by Easton *et al.[131]* This locus is orthologous to the rat *Mcs1b* locus. The human haplotype block associated with risk contains annotated genes, *MAP3K1*, *C5ORF35* and *MIER3*. A recent study found that miRNA targeting of *MAP3K1* significantly suppressed proliferation and invasion of breast cancer cells [215]. Somatic mutations in the cancer genome atlas study found *Map3k1* alterations were enriched in the luminal A subtype of breast cancer. *Map3k1* variations were mainly inactivating frameshift deletions or insertions [216]. However, the exact role *MAP3K1* plays in breast cancer remains to be elucidated. My studies show that *Map3k1* expression increases on DMBA exposure and that *Map3k1* levels are significantly lower in *Mcs1b* resistant MEC extract as compared to susceptible extracts. This makes Map3k1 a strong candidate for the *Mcs1b* locus.

GPBP1 is an uncharacterized gene. It was first identified in atherosclerotic plaques but later a more ubiquitously expressed variant was identified. It functions as a GC-rich promoter-specific transcription factor. Thus this protein may bind DNA and perform transcription factor activities. My experiments show that there is a significant effect of genotype on *Gpbp1* expression. DMBA exposure has the opposite effect on *Gpbp1* expression in *Mcs1b* resistant and WF susceptible rat MEC extracts. DMBA causes an increase in *Gpbp1* expression in *Mcs1b* resistant rat MEC extracts and a decrease in expression in susceptible rat MEC extracts. The significance of this observation is yet unknown and warrants further investigation.

Previous investigations by denDekker *et al.* identified *Mier3* as a candidate gene, but results from my experiments showed no variation in the full-length variant of *Mier3* expression levels between genotypes, with or without DMBA exposure. However, the alternative splice variant identified by denDekker *et al.* was found to follow the same trend that was previously observed for the full-length variant of *Mier3*. I found that the *Mier3-alt5P* was differentially expressed between *Mcs1b* resistant and susceptible MEC extracts both with and without DMBA exposure. Thus, *Mier3* continues to be a candidate gene that is potentially responsible for *Mcs1b* associated mammary carcinoma resistance. It is possible that *Mier3-alt5P* is the main *Mier3* variant present in MECs such that the difference observed by denDekker *et al.* in whole mammary gland tissue is now observed in *Mier3-alt3P* transcripts in MECs. Alternative splicing is one of the most important ways mammals maintain protein diversity. Alternative splicing is used to generate multiple proteins from the same locus. There are two major advantages to alternative splicing, it creates more functional proteins from the same mRNA and it selects the

isoform used in the cell. Including or skipping exons usually alters protein structure and thereby protein functions. Alternative non-coding or partially coding exons usually harbor *cis* regulatory elements that control subcellular localization, stability and translational efficiency of the encoded protein; thereby, controlling protein localization and density. Alternate splicing is usually common during development and in cell lineage differentiation [217, 218]. Although we are aware of the sequence variation of the *Mier3* mRNA isoform, we do not have much information regarding its function.

The congenic animals used differ genetically, only at the *Mcs1b* locus. Resistant female rats differentially express *Mier3-alt5p, Map3k1, Gpbp1* compared to female rats harboring a susceptible WF allele at *Mcs1b*. There seems to be a larger reserve of luminal mammary epithelial cells in *Mcs1b* resistant mammary glands compared to susceptible WF rats. Therefore, any one or a combination of these observed differences could be responsible for *Mcs1b* associated resistance to mammary cancer. More empirical work is necessary to determine the mechanisms by which *Mier3-alt5p, Map3k1* and *Gpbp1* are working to alter mammary carcinoma susceptibility.

Materials and Methods

Generating WF.Cop Congenic Line N3

All animals used for this study were housed by the University of Louisville Research Resources Center Animal Facility. All protocols followed were approved by the University of Louisville IACUC.

WF.Cop line N3 was developed through a backcross of *Mcs1b* resistant line T to WF rats strain. The resultant pups were genotyped using informative markers as decribed in chapter 2 and chapter 3. The list of markers used is provided in table 10. A

Table 10. Informative Makers Used to Genotype Congenic Line N3

Primer and probe Sequences for SNPs in N3 locus					
Samuelson Lab ID	**Type**	**Forward Primer Sequence**	**Reverse Primer Sequence**	**Probe-Copenhagen allele**	**Probe-Wistar-Furth allele**
A12v Allelic Discrimination Assay	SNP	CAACTCATGTTCAGAGACATTTTCCT	TGTGTGGTCTTCTGTTTTCTTGGA	VIC-AGACTTCTCAAACCAG-MGBNFQ	6FAM-AGACTTCTCCAACCAG-MGBNFQ
A12oo Allelic Discrimination Assay	SNP	AACGTTACCGCATTGACACTTG	CAGGCTAGCCTGGGCTATAAGAA	VIC-AAGATTTTTATTTGTTTCAACCAG-MGBNFQ	6FAM-AAGATTTTTATTTGTTTGAACCAG-MGBNFQ
A46-SNP-A Allelic Discrimination Assay	SNP	GCACTCCAGTGGTAAGACTAAAGCA	TTTACCTGGAAACGGAATGCA	VIC-AGAACTTCTACAGAACCT- MGBNFQ	6FAM-AGAACTTCTACAGGACCT-MGBNFQ
A74-SNP-17 Allelic Discrimination Assay	SNP	CTGGGTCAACAACTCGCTGAT	GGAACGTAAGAAACGCTGTAGAGAT	VIC- AGCTGCAAATGTC-MGBNFQ	6FAM-AGCTGCACATGTCAT-MGBNFQ

This table displays the primer sequences along with probe sequences used to genotype *Mcs1b* rats.

recombinant rat with the Cop allele represented in the congenic line N3 was selected and further backcrossed to WF females to expand the line. Rats that are heterozygous Cop at the N3 locus were inbred to generate rats that were homozygous Cop at the N3 locus. Congenic line N3 was then fixed and rats were used for experiments. Females were administered DMBA at fifty to fifty five days of age and were euthanized at twelve weeks of age. Cervical, abdominal and inguinal mammary glands and spleens were collected postmortem.

Isolation of Mammary Epithelial Cell Enriched Extract

MEC extract was prepared as previously described [210]. For each rat, both abdominal and inguinal mammary glands without lymph nodes were excised and kept separately in GIBCO DMEM/F12 media (Invitrogen). These mammary glands were scissor minced and digested in 10 ml of DMEM/F12 containing 0.01 g/ml of type III Collagenase (Worthington) for 2 hours at 37˚C with gentle horizontal shaking. 0.2 mg/ml of DNase I (Worthington) was then added to this digest for ten minutes under vigorous shaking. This extract was then centrifuged and the supernatant fat was discarded. The pellet was then washed with DMEM/F12. The cells were monodispersed by dissolving the pellet in 2 ml of Hank's balanced salt solution containing 0.025% trypsin and 6.8 mM EDTA. This reaction was stopped after 5 minutes by adding 4 ml of DMEM/F12 containing 10% fetal bovine serum (FBS). The cells were centrifuged and resuspended in 2 ml of DMEM/F12 containing 10% FBS and passed through a 40 μm cell strainer. The centrifuge tube was rinsed with another 2 ml of DMEM/F12 containing 10% FBS and passed through the same 40 μm cell strainer to collect a single cell suspension of MEC extract.

Antibody Staining and Flow Cytometric Analysis of MEC Extract

MEC extract was stained as previously described [210]. Single cell suspensions were stained using antirat CD24, CD29, CD31 CD45 and live/dead stain (BD Pharmingen) antibodies. The MEC extract was centrifuged and suspended in phosphate buffer saline (PBS). Cells were stained with 0.5 µg of each antibody per 10^6 cells in 200 µl PBS for 30 minutes at 4˚C in the dark. The volume was brought up to 1 ml and centrifuged. The cells were washed and later fixed using fixation buffer (1 part 4% formaldehyde to 3 parts PBS) for 10 minutes at room temperature. Samples were washed and resuspended in 600 µl PBS. Cells were counted using a flow cytometer and analyzed using FACS Diva.

Gene Expression Assays

RNA was isolated from the MEC extract using the MagMAX^{TM} -96 Total RNA isolation kit (Ambion). Total RNA was quantified using Nanodrop 1000 (Fisher Scientific). cDNA was synthesized using 1µg total RNA, 0.5x RNAsecure, 5µM random hexamers, 25ng/µL oligo(dT18), and 0.5 mM dNTPs in a final volume of 20µL. The reaction mixtures were incubated for 5 minutes at 65º C. Then 1× first strand buffer, 100mM DTT, and 1µL Superscript III (Life Technologies) was added to each reaction mixture. Reactions were incubated 5 m at 25º C, 1 h at 50º C, and 15 m at 70º C. *Taq*Man QPCR primers and MGB probes (Applied Biosystems) (sequences are in table 11), designed by denDekker *et al.* [137], were used in a 20µl *Taq*Man QPCR reaction. Each reaction comprised of 1× *Taq*Man Buffer A (Applied Biosystems); 3.5 mM MgCl2; dATP, dCTP, dGTP, and dTTP at 200 µM each; experimental primers at 500 nM each; 200 nM *Taq*Man experimental probe (Applied Biosystems) and 0.4 units of *Taq* Gold

Table 11. List Primers and Probes Used for Expression Analysis in *Mcs1b* Rat MEC Extracts

Rat *Mcs1b* Target and Rplp2 Primers and Probe Sequences Used for QPCR			
Assay Name	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')	Probe Sequence (5' to 3')
Rplp2	TGAACGACTCAACAAGGTCATCA	CAACACCCTGAGCGATGACA	VIC-CTGAATGGAAAGAATATTGAG
Map3k1	TCCTCATATTGTCAGTACCGATGTC	TTGCAAGGCAAAGGCTAAGAG	6FAM-CCTGTTGAAATCAGGTATAA
Mier3	CGAAAGGTACTGCTGTAATGGAAA	GCACTCCTCTTCAGTCCAAGCT	6FAM-CGTCTCAAGAAGGAATG
Gpbp1	GAGTAGAAGAGGAGCATGAAGATGAA	TGGTGAGTACTATTGCTGTTATGCAA	6FAM-CTCAGAGAAGGATGACGAC
Mier3-Alt5P	ATGGCGGAGGCTTCCTTT	TCAAAATCATGATCCTCAGAAGACA	6FAM-AGCCCAGTTGGGTCT

This table displays the primer and probe sequences used for gene expression analysis on *Mcs1b* MEC extracts. The genes analyzed are *Rplp2, Map3k1, Mier3, Gpbp1* and *Mier3-Alt5P*.

DNA Pol (Applied Biosystems). In case of *Rplp2,* primers were at the concentration of 125 nM each and 200 nM rodent *Rplp2* probe was used. Real-time QPCR was run on an Applied Biosystems Step One Plus real-time PCR machine. Real-time QPCR was run at 50°C for 2 min and 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The fluorochrome FAM was used on target gene probes and VIC was used on the rodent *Rplp2* probe (Applied Biosystems). Analysis was performed by the delta-delta CT method using Rplp2 as the internal control. Each sample measurement is an average of three technical replicates. Data were analyzed by performing a two way ANOVA and *post hoc* t-tests.

Immunofluorescence of Transverse Sections of Rat Mammary Glands

Rat right cervical mammary glands were isolated and fixed in zinc fixative for 16-24 hours. The mammary glands were then transferred into 70% ethanol. The glands were then submitted to the UofL pathology core for paraffin embedding and tissue sectioning. Each tissue section was deparaffinized and rehydrated by transferring it successively into decreasing concentrations of alcohol. The tissue was rinsed in water and immersed in 10mM tris EDTA and microwaved on high for 20 minutes. The tissue was allowed to cool at room temperature and rinsed in PBS. The tissue section was blocked with PBS containing 0.5% triton, 5% normal donkey serum. The blocking reagent was tapped out of the slide which was then incubated with primary antibody (rabbit polyclonal Mier3-ab69877- abcam, negative control for rabbit IgG Ab-1- thermoscientific, FITC conjugated Peanut lectin (PNL)-sigma L7381, Anti-Rat CD90 BD Pharmigen) in PBS overnight at 4˚C. The tissue was washed with PBS. The tissue was then incubated with secondary antibody (donkey anti-rabbit Alexa Fluor 546 conjugate, ThermoFisher

Scientific) in PBS at room temperature for 1-1.5 hours. The tissue was washed with PBS and was permanently mounted. The tissue was visualized using a confocal microscope and representative images were taken.

Genomics and Statistical Analysis

Quantitative PCR (QPCR) data were analyzed using the delta-delta CT method using *Rplp2* as the internal control. qPCR data were analyzed by performing a two way ANOVA and *post hoc* t-tests. Independent variables for comparing mammary gland transcript levels were genotype and DMBA exposure. The flow cytometry data were analyzed using a two way ANOVA. Data were transformed prior to analysis by taking the square root of the ratio of the cell count to the gated parent population. Genotype and DMBA exposure were independent variables**.**

CHAPTER V

CONCLUSION

Breast cancer is a complex disease characterized by its environment, genetic and epigenetic components. Many approaches have been used to study the mechanisms associated with breast cancer, each with their own advantages and disadvantages. With every study we gain new insight into this multifaceted collection of diseases known as breast cancer. The laboratory rat has been used extensively to study breast cancer. There are several rat genomic loci that associate with breast cancer susceptibility. This study focused on two such loci, namely *Mcs3* and *Mcs1b*, using congenic rats as a model of breast cancer susceptibility.

The *Mcs3* locus was initially predicted across two peak markers *D1Mit11* and D1*Wox6* on rat chromosome *1* [173]. Rat *Mcs3* had not been physically confirmed; and thus, its location on chromosome *1* hadn't been fine-mapped. In this study, the *Mcs3* locus was physically confirmed and mapped to 25.8 MB region on rat chromosome *1* using multiple congenic lines. *Mcs3* is highly relevant as it contains five genes and 1 SNP that have been associated with breast cancer susceptibility. Bioinformatic analysis of the locus and it orthologous region in humans led to the identification of 3 candidate genes *PAK1*, *ILK* and, *IL18BP*. Among these five genes *PRC1*, *PAK1*, *ILK* and *IL18BP* are expressed both in rats and humans. *PAK1* downstream pathways play an important role in development and maintenance of metastatic phenotype in breast cancer cells. *PAK1* was

also shown to be a predictor of recurrence and tamoxifen resistance in post-menopausal breast cancer [198]. *ILK* may be an important marker since increased expression of *ILK* is associated with aggressive progression and poor prognosis of breast cancer [200]. *IL18BP* regulates the activity of interleukin 18. *IL-18* expression is significantly higher in malignant breast tumor tissue as compared to breast tissue of patients with benign breast disease [202].

Overall, the *Mcs3* congenic rats provide an experimental organism which could be used in the future to perform functional genetic studies which can be translated to human breast cancer. Studying the *Mcs3* locus is highly relevant because the exact mechanism by which *Mcs3* contributes to mammary carcinoma susceptibility is not known. Any of the three candidate genes mentioned above could be responsible for the reduced mammary carcinoma susceptibility associated with *Mcs3* or the *Mcs3* locus could contain a novel breast cancer susceptibility gene.

In comparison to *Mcs3*, considerable research had been done on the *Mcs1b* locus prior to my studies involving this QTL. The *Mcs1b* locus had been physically confirmed and fine mapped [181]. It was known that the *Mcs1b* locus acted in a mammary gland independent manner and full-length *Mier3* variant was predicted as the *Mcs1b* candidate gene [137]. It was also known that *Mier3* was expressed in mammary epithelial cells surrounding mammary ducts. There was a *Mier3* splice variant with an alternate 5' start site (*Mier3-alt5P*) that was more highly expressed in *Mcs1b* mammary glands compared to susceptible mammary glands [137]. One of the goals of this study was to characterize *Mcs1b* mammary epithelial cells with respect to proportion of luminal and basal epithelial cells and expression of full-length *Mier3, Map3k1, Gpbp1* and *Mier3-alt5P* compared to

DMBA induced mammary carcinoma susceptible WF rats. This study was performed on MEC extracts that were enriched for mammary epithelial. MEC extracts were obtained from mammary glands by enzymatic digestion. The MEC extract was then aliquoted for antibody staining and cytometric analysis, and qPCR expression analysis assays. In cytometric analysis, single cells were identified from the population based on forward and side scatter widths. Dead cells were gated out of the single cell populations by using a viability stain. Cells that were CD45+ and CD31+ were gated out, as these were hematopoietic and endothelial cells, respectively. Next, the selected cell population was separated into basal and luminal cell populations, based on CD29 and CD24 expression levels. CD24+CD29hi are the basal cell population while CD24+CD29med are the luminal cell population

Findings from this study indicate, the proportion of luminal MECs was higher in *Mcs1b* resistant MEC extracts compared to susceptible MEC extracts. The significance of this finding is unknown. However, this observation suggests that luminal MECs are potentially the target cells of DMBA induced carcinogenesis and future mechanistic studies could be conducted in luminal MECs. Expression assays on MEC extracts suggest that *Map3k1, Gpbp1* and *Mier3-alt5P* are potential *Mcs1b* candidate genes [137]. Rat *Map3k1, Gpbp1* and *Mier3-alt5P* transcripts were expressed at a significantly lower level in *Mcs1b* resistant MEC extracts as compared to susceptible MEC extract without DMBA exposure. Both *Map3k1* and *Mier3-alt5P* continued to remain expressed at a significantly lower level in *Mcs1b* resistant MEC extracts as compared to susceptible MEC extract with DMBA exposure. There was a significant interaction between genotype and DMBA exposure for *Gpbp1* expression. The susceptible WF and Mcs1b rats only differ in their

genome at the Mcs1b locus. The results suggest that *Map3k1, Gpbp1* and/or *Mier3-alt5P* are responsible for the *Mcs1b* associated reduced susceptibility to DMBA induced carcinogenesis.

MAP3K1 is a serine/threonine kinase with a role in ERK and JNK kinase pathways, as well as the NF-kappa-B pathway. Breast cancer susceptibility associated SNP *rs889312* identified by Easton *et al*. is orthologous to the rat *Mcs1b* locus and nominates *MAP3K1, C5ORF35* and *MIER3* as breast cancer susceptibility candidate genes [131]. A recent study found that miRNA targeting of MAP3K1 significantly suppressed proliferation and invasion of breast cancer cells [215]. *GPBP1* is an uncharacterized gene. It's predicted function is as a GC-rich promoter-specific transcription factor. Thus, Gpbp1 may bind DNA and perform transcription factor activities. Previous investigations by denDekker *et al*. identified *Mier3* as a candidate gene, but results from my experiments showed no variation in full-length *Mier3* expression levels between genotypes, with or without DMAB exposure. The full-lengthMier3 was the variant tested by denDekker et. al. However, the alternative splice variant *Mier3-alt5P* was found to follow the same trend that was previously observed for *Mier3*. It is possible that *Mier3-alt5P* is the main *Mier3* variant expressed in MECs. The *Mier3-alt5P* is predicted to include a casein kinase phosphorylation site at the N-terminus compared to *Mier3* protein. Although we are aware of the sequence variation of the *Mier3* mRNA isoform, we do not know its function. Therefore, *Map3k1, Gpbp1* and/or *Mier3-alt5P* are *Mcs1b* candidate genes and the luminal mammary epithelial cell population is the likely target of DMBA carcinogenesis.

Future Directions

In conclusion, *Mcs3* was physically confirmed and mapped to a 25.8 MB region on rat chromosome *1* and *PAK1, ILK* and *IL18BP* are potential candidate genes. The next step for *Mcs3* would be to identify if *Mcs3* acts in a mammary gland independent manner. Once that is completed, expression studies can be performed on the candidate genes while continuing congenic studies to further fine map the *Mcs3* locus in search of potential novel breast cancer susceptibility genes.

This study suggests the luminal mammary epithelial cell population is a target cell population of DMBA carcinogenesis in *Mcs1b* congenic rats, and that *Map3k1, Gpbp1* and/or *Mier3-alt5P* are *Mcs1b* candidate genes. This should be further investigated by isolating luminal mammary epithelial cells and performing expression assays to quantify *Map3k1, Gpbp1* and/or *Mier3-alt5P* in *Mcs1b* resistant mammary glands and compare them to susceptible mammary glands.

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CURRICULUM VITAE

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Education

University of Louisville, Louisville, KY **August 2010-May 2016**

PhD, Biochemistry and Molecular Genetics

Mentor: David Samuelson, PhD

University of Louisville, Louisville, KY **August 2010- May 2015**

MS, Biochemistry and Molecular Biology, GPA: 3.99

Mentor: David Samuelson, PhD

Mumbai University, Mumbai, India **August 2008- June 2010**

MS, Biochemistry and Molecular Biology

Mumbai University, Mumbai, India **August 2005- June 2008**

BS, Double Major in Life Sciences and Biochemistry

Personal Statement

I come from a family which has instilled in me the drive and passion to be a better and compassionate human being. Due to lack of opportunities and difficulties, my parents had to compromise on their choices and hence, have always wanted the best for me, motivating me to aim higher and allowing me to pursue my calling.

I had a good academic record throughout my years in school and college, with a steady improvement curve over the years. I was always amongst the top 5% students during my undergraduate and graduate education. The undergraduate curriculum at Sophia College, Mumbai provided me with strong back ground in Life Sciences and Biochemistry. During my undergraduate I studied a wide variety of subjects which included Physics, Immunology, Developmental Biology, Biotechnology, Genetics, Cell Biology, Bio-Physical Chemistry, Clinical Biochemistry, Industrial Biochemistry, Bioinformatics and other topics related to Biochemistry and Life Sciences taught by highly qualified and competent faculty members and frequent guest lecturers.

My dissertation topic during my Masters in India was 'The effect of local varieties of plant growth promoters on *Vigna radiata'*. The project work helped me greatly in sharpening my research abilities and learning various techniques and its practical application via research. I soon fully grasped that biochemistry is an interdisciplinary field offering vast opportunities for research. Right from testing the chemical secrets of fertility, to genome sequencing, devising ways to improve immunity against diseases like cancer and AIDS to discovering ways to extend the human life span, the list is large.

I came to the United States of America to pursue my Ph.D. in Biochemistry and Molecular Biology. I am currently a graduate student working in Dr. David J. Samuelson's lab. **'Genetic and environmental breast cancer susceptibility' is the focus of the lab**. My graduate work is mainly focused on *Mammary Carcinoma Susceptibility Locus 3 (Mcs3) Mcs3* is a QTL predicted on rat chromosome*1*. It was identified in a linkage analysis study and was predicted to reduce mammary carcinoma susceptibility in rats. I have physically confirmed the *Mcs3* locus and I am currently working on fine mapping this locus to identify its physical location on rat chromosome*1*. The other half of my dissertation project deals with another QTL- *Mcs1b*. Previous studies from Dr. Samuelson's lab identified *Mier3* as a potential candidate gene which could be responsible for the altered mammary carcinoma susceptibility observed in rats with the *Mcs1b* Copenhagen (Cop) allele compared to the Wistar Furth (WF) allele. It is also known that the *Mcs1b* locus acts in a mammary gland autonomous manner. My work tests the hypothesis that *Mcs1b* rat mammary epithelial cells differentially express *Mier3* compared to the susceptible WF rats.

Positions

08/2010 – 04/2016	Graduate Research Assistant, University of Louisville
08/2014 – 05/2015	Graduate Student Representative at the SOM Medical council of University of Louisville

08/2012 – 05/2013	Representative of the Department of Biochemistry and Molecular Biology in the Graduate Student Council of University of Louisville
08/2011 – 05/2012	Representative of the Department of Biochemistry and Molecular Biology in the Graduate Student Council of University of Louisville
08/2011 – 12/2011	Teaching Assistant, University of Louisville

Honors

1) Integrated Programs in Biological Sciences Fellowship from 8/2010 to 7/2012.

2) Received the Gloria D' Souza scholarship from Sophia College for 'Academic Performance and Responsible Involvement' in the year 2008.

3) I was part of the 'Chem Club' in Sophia College, Mumbai, India in the year 2006-2007.

4) I was an organizer for 'Build it Up' which was hosted by the Dept. of Biochemistry for 'Ananya 2008' [Science festival at Sophia College, Mumbai, India]

Seminars and Workshops Attended

1) Attended the Breast Cancer and the Environment Research Program's Extended Environmental Exposures Annual Meeting to be held in Cincinnati in 2011.

2) Attended the lecture workshop "150 yrs since Darwin- Behavioral Adaptation and Evolution" on August 29th-30th, 2008 by Science Academy education program INSA Delhi, ISA Bangalore, NASI Allahabad.

3) Attended lecture workshop- Frontiers in neuroscience on January 4th-5th, 2008 by Science Academy education program INSA Delhi, ISA Bangalore, NASI Allahabad.

4) Visited Advanced Center for Treatment, Research and Education in Cancer (ACTREC) on an educational trip organized by Sophia College 2007-2008.

Publications

Le S, Samuelson DJ. Mammary Carcinoma Susceptibility locus, *Mcs3*: Physical confirmation and fine mapping. Manuscript writing is in progress.

The rest of my work will be added to another paper with other authors.

Posters

1) Saasha Kareparembil, David Samuelson. A 29MB region of Mcs3, a mammary carcinoma susceptibility QTL, reduces mammary carcinoma susceptibility. Research Louisville 2015.

2) Saasha Kareparembil, David Samuelson. A 29MB region of Mcs3, a mammary carcinoma susceptibility QTL, reduces mammary carcinoma susceptibility. Biochemistry Retreat 2015.

3) Saasha Kareparembil, Aaron Puckett, David J. Samuelson. Predicted mammary carcinoma susceptibility QTL on *RN01* named *Mcs3* contributes to reduced mammary carcinoma susceptibility in WF.Cop congenic rats BMB retreat 2013

4) Saasha Kareparembil, Aaron Puckett, David J. Samuelson. Predicted mammary carcinoma susceptibility QTL on *RN01* named *Mcs3* contributes to reduced mammary carcinoma susceptibility in WF.Cop congenic rats BMB student recruitment 2013

5) Saasha Kareparembil, Aaron Puckett, David J. Samuelson. Predicted mammary carcinoma susceptibility QTL on *RN01* named *Mcs3* contributes to reduced mammary carcinoma susceptibility in WF.Cop congenic rats Research Louisville 2013

6) Anna Thaman, Saasha Kareparembil, David J Samuelson DJ. Breast Cancer Susceptibility Genes: Characterizing MIER3. Research Louisville 2014

Research Support:

Integrated Programs in Biological Sciences Fellowship from 8/2010 to 7/2012

Research Experience:

Graduate Research: Department of Biochemistry and Molecular Genetics, University of Louisville
Mentor: Dr. David Samuelson
Dissertation Project: Genetics and Molecular Analysis of Rat mammary cancer susceptibility
From 03-2011 to Spring 2016 (projected graduation date)

Worked extensively with rats:

- Learned how to breed rats and maintain a rat colony
- Learned how to genotype rats
- Administered carcinogen 7 12-dimethylbenz(a)anthracene (DMBA) to rats through oral gavage and determined mammary tumor number using surgery
- Learned how to isolate rat mammary glands and isolated mammary epithelial cells
- Learned to isolate granulocytes from rat blood and perform transwell assays

General laboratory techniques learned:

- PCR methods including RT-QPCR
- DNA and RNA extraction
- Flow cytometry
- Maintaining a cell culture
- miRNA estimation
- Immunohistochemistry
- Western blots
- Immunofluorescence

PhD DISSERTATION COMMITTEE

Dr. David Samuelson (Mentor)
Dr. David Hein
Dr. Ronald Gregg
Dr. Alan Cheng
Dr. Christine Schaner-Tooley

REFERENCE

Dr. David Samuelson (PhD dissertation mentor, University of Louisville)
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