ROLE OF TUMOR SUPPRESSOR DMP1 IN THE INITIATION AND PROGRESSION OF BREAST CANCER

BY

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Acknowledgments

The work in this dissertation presents a modest portion of my parent’s life-long and ingenious efforts to provide a fruitful environment for the pursuit of my personal endeavors. It began with the earliest memory of my parents, sternly but uneasy, asking “Hočeš li da kopaš kanale čitav život?” (English Translation: “Do you want to dig ditches for the rest of your life?”), which persuaded me to keep afloat in school until I discovered my passion for science. In 2000, they made a difficult decision to immigrate to United States knowing that my long-term goals were unattainable living in a society recovering from the devastating Balkans war. Their sacrifice to leave the entire family and move to the “New World” not only provided an opportunity for me to complete a PhD degree but also gave my younger brother a chance to pursue his dreams in medicine. I know that our hard work is all the gratitude that you will ever need but it is certain that your influences will be the primer for our life-long success.

Special appreciation goes to my peers and classmates (Andy, Paul, Phil, Ryan, Peter, Dave, and Daniel) whose participation in daily lunch discussions almost solely contributed to the “Philosophy” portion of my PhD degree. Our conversations worked through tough political and religious issues as well as planning of several first-in-class charity events including Peter’s fictitious wedding. The relationships with other friends at Wake Forest and in Winston-Salem provided for my personal growth in many different aspects. Certainly, I will continue to cherish all the memories (good and bad) for the rest of my life.
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<tbody>
<tr>
<td>a.a.</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ABI</td>
<td>Applied biosystems inc.</td>
</tr>
<tr>
<td>ACS</td>
<td>American cancer society</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>CA-MEKK1</td>
<td>Constitutively active MEKK1</td>
</tr>
<tr>
<td>Cas</td>
<td>CRISPR-associated</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf-intestinal phosphatase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeat</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DMBBA</td>
<td>Dimethylbenzanthracene</td>
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<tr>
<td>DMP1</td>
<td>Cyclin D-binding myb-like protein 1</td>
</tr>
<tr>
<td>DMTF1</td>
<td>Cyclin D-binding myb-like transcription factor 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>DOD</td>
<td>Department of defense</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactosidase</td>
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<tr>
<td>gDNA</td>
<td>Genomic deoxyribonucleic acid</td>
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<tr>
<td>HA-tag</td>
<td>Hemagglutinin tag</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human mammary epithelial cells</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MAP3K1</td>
<td>Mitogen activated protein kinase kinase kinase 1</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEKK1</td>
<td>Mek kinase 1</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>M.W.</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NCI</td>
<td>National cancer institute</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>OIS</td>
<td>Oncogene-induced senescence</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase M2</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>Pten</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qLOH</td>
<td>Quantitative loss of heterozygosity</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>S</td>
<td>Serine</td>
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<tr>
<td>SASP</td>
<td>Senescence-associated secretory phenotype</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>------------------------------------------------------------------</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEAP</td>
<td>Secreted endocrine alkaline phosphatase</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short-interfering ribonucleic acid</td>
</tr>
<tr>
<td>SP</td>
<td>Serine-Proline</td>
</tr>
<tr>
<td>SRSF</td>
<td>SR splicing factors</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TN</td>
<td>Triple negative</td>
</tr>
<tr>
<td>TP</td>
<td>Threonine-Proline</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WFUHS</td>
<td>Wake Forest University Health Sciences</td>
</tr>
<tr>
<td>YY1</td>
<td>Ying Yang 1</td>
</tr>
<tr>
<td>4-HT</td>
<td>4-Hydroxytamoxifen</td>
</tr>
</tbody>
</table>
Abstract

Maglic, Dejan

ROLE OF TUMOR SUPPRESSOR DMP1 IN THE INITIATION AND PROGRESSION OF BREAST CANCER

Dissertation under the direction of

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Breast cancer is the second most common cause of cancer related death in women. Decades of research have demonstrated complexity and high heterogeneity of this disease. The tumor heterogeneity is a result of many altered genomic, epigenomic, and protein modification states that contribute to a distinctive proliferative advantage. The same deregulations that initiate tumorigenesis of mammary epithelial cells are believed to coerce therapeutic resistance. On the other hand, population-wide screenings have increased diagnosis of women with breast cancers without specificity for the tumors with an aggressive phenotype. Hence, identification of the cancer cell’s intrinsic alterations that faithfully predict tumor progression has a potential to significantly improve their clinical management.

To understand stepwise breast cancer initiation process, we first investigated the Erbb2-driven senescence in mammary premalignant lesions and the mechanism of evasion during the malignant progression. We identify the Dmp1 transcription factor as a
central sensor of the oncogenic stress stemming from Erbb2 to activate the p53 tumor suppressor pathway. In the MMTV-Her2/neu mouse model of breast cancer, hemizygous Dmp1 deletion was sufficient to alleviate the p53-dependent senescence program. Similarly, the DMP1 locus was haploinsufficient for tumor suppression in ~42% of human breast cancers with mutual exclusiveness with inactivation of the INK4A/ARF or p53. Importantly, patients with DMP1 loss maintained wild-type p53 and had better clinical outcome than the patients with intact DMP1 loci. The haploid insufficiency of DMP1 in breast cancer was found in part due to aberrant splicing that downregulates the tumor suppressor, DMP1α, while upregulating the tumor-promoting DMP1β isoform. DMP1β tumor-promoting phenotype observed in human breast cancer was recapitulated in the mouse mammary epithelium. Aside from Erbb2, we also identify MEKK1 as a novel post-translational modulator of the Dmp1 protein, which activated the Dmp1-Arf-p53 and induced cellular senescence. In human breast cancer, the MEKK1 locus was hemizygously deleted in ~20% of the cases and its expression predicted clinical outcome.

Overall, our work demonstrates a critical role of DMP1 in preventing transformation of the mammary epithelium, which was bypassed with combination of selective deletion and aberrant splicing of the DMP1 locus to support breast cancer progression.
CHAPTER I

General Introduction

Dejan Maglic

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This unpublished chapter was composed by Dejan Maglic with an editorial assistance of Guangchao Sui.
I.1. Breast cancer in the era of high-throughput analyses

Breast cancer, excluding basal cell carcinoma of the skin, is the most common tumor type in women. It is predicted that in 2013 approximately 40,000 women in United States will die as a result of breast cancer progression (1). In the past several decades, identification of the tumor drivers and their use as prognostic indicators have significantly improved patient outcome. Deregulation of the hormone receptors, specifically the Estrogen receptor alpha (ERα), in mammary epithelium was recognized as the most important molecular driver of breast cancer development and progression. Indeed, ~70% of breast cancers overexpress ERα and depend on its action during the disease progression, which incited a successful development of the first targeted agent for cancer therapy (2). The molecular function of the other breast cancer-associated hormone receptor, Progesterone receptor (PR), except its role as a clinical marker, remains unclear and it is a subject of many current investigations (3-4). The anti-estrogen therapies constituting of the selective estrogen receptor modulators (SERMs) or those that inhibit aromatase, a key estrogen-synthesizing enzyme, have proven useful for the treatment of hormone receptor positive tumors (5). More recently it was discovered that ~25% of breast cancer patients have amplification or overexpression of ERBB2 (HER2) proto-oncogene in the tumor tissues which was found to be a useful clinical marker for poor patient outcome (6). The ERBB2 is a member of the epidermal growth factor receptor (EGFR) family whose activation is independent of specific ligand binding but promoted by heterodimerization with other members of the EGFR family. Development and clinical implementation of the anti-HER2 monoclonal antibody, trastuzumab (Herceptin®), significantly improved the outcome of the patient population
with HER2 positive tumors (7-9). Nevertheless, many breast cancer patients receiving an anti-estrogen or the anti-HER2 therapies develop tumor clones that are refractory to the first-line therapy (10-11). In addition, approximately 15-20% of breast cancers lack expression of the hormone or HER2 receptors and are designated as triple negative (TN). The breast cancer patients in this population have significantly worse clinical outcome due to lack of effective targeted therapies for the treatment (12). Thus, breast cancer remains a significant health care burden in developed countries as only a select patient population has benefited from the improved therapies over past several decades. On the other hand, implementation of mammography screening for early detection was recently re-evaluated and found to result in ~30% overdiagnosis of breast cancer without a significant improvement in long-term patient survival (13). Detection and treatment of the indolent tumors, which would otherwise be left without any intervention and clinically remain asymptomatic during the patient’s lifetime, contribute to a significant psychological stress, lower quality of life, and overall increase of health care cost. Importantly, this finding supported a growing paradigm that balance between early cancer detection and therapeutic intervention guided by reliable markers will define success of future management of cancer patients. Additional research will be necessary to define new markers that reliably differentiate indolent from aggressive breast tumors.

A recent boom in gene expression and deep sequencing platforms has revolutionized unbiased approaches to identify epigenetic and genetic alterations in the tumor tissues. In a seminal study using a microarray gene expression analyses, Perou et al reclassified breast tumors into five new molecular subtypes based on the intrinsic gene expression signatures (14). The new breast cancer subtypes encompass Luminal A or
Luminal B (tumors expressing luminal cell markers), Basal-like (tumors with a signature of mammary basal cells), HER2-enriched (tumors with activation of signaling molecules within the HER2 pathway), and normal-like tumors (tumors with gene signatures resembling normal mammary glands) (14). Importantly, this new classification was adopted by many clinics as it better predicted patient outcome independent of other prognostic variables (15). The luminal subtypes were found to commonly express ER and PR; however, Luminal A tumors have lower proliferation index and are a predictor of better overall survival than the Luminal B subtype (16). The HER2-enriched tumors have an aggressive phenotype, but due to the patients’ eligibility for the trastuzumab therapy, the clinical outcome of this group has significantly improved in the past two decades. The normal-like breast tumors make up the smallest subtype (>10%) with lack of ER/HER2 gene signatures but resemble those of adipose tissue. Due to low incidence and poor characterization, very little is known about the normal-like breast tumors. In fact, several groups have suggested that the adipose gene signature observed in the normal-like subcategory is a result of poor breast tissue sampling during biopsies and thus the normal-like tumors misrepresent other subcategories (17). Lastly, the basal-like breast tumors have the worst prognostic outcome among all the subtypes in part due to frequent \textit{p53} mutation and lack of other molecular targets (i.e. ER, PR, and HER2) making them resistant to the existing chemotherapies or ineligible for the targeted therapies (18-19). Moreover, an extension of the original microarray studies identified another subcategory named as claudin-low, which clustered closely with the basal-like molecular subcategory. The claudin-low tumors were characterized by low expression of the cell adhesion proteins, including \textit{claudin} genes, and gene signatures resembling the
mammary stem cells and those of an immune response. Breast cancer patients with the claudin-low tumor signature also have poor clinical outcome without any standard therapeutic approach (20-21). Another molecularly defined subcategory of breast cancer designated as apocrine carcinoma, which lacks both hormone receptors but is positive for the androgen receptor (AR) and HER2 expression, has no standardized therapy (22-23). Work from the Myles Brown lab has demonstrated a contribution of the AR-Myc-Her2 axis to a positive feed-forward signal necessary for the AR-driven oncogenic growth (24-25). Hence, androgen-suppressing therapies used in the standard therapy for prostate cancer could be effective in treatment of the apocrine breast cancers.

Aside from gene expression profiling, high-throughput and deep genomic sequencing of the tumors and their matched normal tissues have provided a wealth of information on the involvement of different pathways in each molecular subcategory of breast cancer. In particular, this strategy confirmed frequent alteration of known cancer-associated pathways (p53, RB, and PI3-Kinase) and the genes (*MAP3K1, TBX3, CDKN1B, and GATA3*) previously unrecognized for their association with breast tumorigenesis (26-28). Importantly, it further provided support for the recognition that massive heterogeneity within breast tumors contributes to many more disease subcategories than previously believed. The simultaneous alteration of multiple tumor-driving pathways offers the rational for future of multi-targeted therapies. Hence, investigative utility of these unbiased approaches was the evidence that success of future therapies will heavily be contingent on personalizing targeted agents. However, it will be necessary to first delineate precise contribution of each alteration and their interplay during the initiation and progression of the disease.
Selection of proper models to investigate driver mutations and their cooperation with other signaling hubs will be essential to translate the genomic findings into clinical applications. The relative quickness of *in vitro* and xenograft models has been instrumental for identification of molecular targets in many pre-clinical studies; however, targeting the identified molecules often fails to show efficacy during the Phase II and Phase III studies in human subjects. Both of the most commonly utilized models lack additional components within the native niche that allows tumors to grow as a functioning organ. Current evidence supports the utility of genetically engineered mice trumps other models when recapitulating a human tumor phenotype (29-30). Indeed, it was recently reported that a well-known oncogenic function of fetal pyruvate kinase, PKM2, failed to recapitulate an established dogma in the mouse model of breast cancer. The loss of PKM2, an essential driver of aerobic glycolysis in cancer cells, unexpectedly accelerated the mammary tumor progression (31). Therefore, development of drugs targeting PKM2 based on the *in vitro* and xenograft data would have failed in breast cancer clinical trials. The establishment of novel genetically engineered mice by the conventional methods is a tedious and costly process and thus prohibits rapid investigation of tumor-associated alterations identified in high-throughput analyses. In particular, understanding cooperation of alterations between multiple pathways is even more complicated as mice with compound genetic alterations require complex breeding schemes. Recent emergence of a novel methodology using the CRISPR (clustered regularly interspaced short palindromic repeat) and Cas (CRISPR-associated) nucleases to efficiently and specifically edit mammalian genome has significantly improved the methodology for establishing genetically engineered mouse models (32-33).
CRISPR/Cas is an evolutionary conserved adaptive immune mechanism in the type II bacteria that mediates sequence-specific cleavage of foreign plasmids or bacteriophage RNA (34). This mechanism has now been adapted to generate mutant mice as a result of Cas9 nuclease-driven double-stranded breaks followed by an error prone non-homologous end joining (NHEJ). Intriguingly, this system can be multiplexed to simultaneously generate mice with mutations in multiple genetic loci (35). Use of this improved methodology and other forthcoming techniques will be instrumental in future mechanistic investigations delineating contributions of each cancer-associated aberration in mammary tumorigenesis.

1.2. Alternative splicing and cancer

Regulation of human genome is a highly complex process and remains an area of active investigation in various normal and disease states. Human tumors have also been recognized to gain a capacity to modulate their genome via multiple mechanisms. Besides genomic mutations, deletions, fusions or epigenetic alterations, deep RNA sequencing has revealed another level of complex misregulation by cancer cells, which alter splicing machinery to aberrantly express variety of tumor-promoting isoforms (36-38). Alternative splicing is a mechanism of encoding multiple functionally distinct proteins from a locus via splicing-driven exon inclusion or exclusion. It is believed that up to 95% of human genome undergoes alternative splicing thus allowing expression of several folds more proteins or peptides compared to number of gene loci (39). This evolutionary conserved mechanism depends on the RNA-binding proteins known as splicing factors, which directly interact with consensus sequences within exons or
surrounding introns (40). The binding of splicing factors to pre-mRNA functions to promote inclusion or exclusion of specific exons. Although alternative splicing was originally described as a functional process in normal development, recent reports suggest strong association of cancer progression with aberrant splicing to express a particular isoform. Involvement of the aberrant splicing in cancer was further confirmed when several splicing factors were found to act as proto-oncogenes. Specifically, SF2/ASF (SRSF1) splicing factor was amplified and frequently overexpressed in human breast tumors (41). Overexpression of SF2/ASF promoted transformation of mammary epithelial cells in vitro and thus provided a strong evidence of its oncogenic role (42). Other splicing factors such as SRSF5, SRSF6, hRNPA2/B1, and Tra2β are upregulated in a variety of tumor types and their expression significantly correlated with tumor progression (43-46). The specific mechanism of splicing factor misregulation in tumor tissues is largely undefined. Phosphorylation by upstream kinases such as AKT in case of SF2/ASF activation is a potential mechanism of splicing factor deregulation in the tumors; however, other mechanisms should also be investigated (47). Furthermore, defining how deregulated splicing factors contribute to cancer initiation and progression is essential to understanding a selective pressure driving the aberrant splicing. Although studying aberrant splicing remains difficult due to mostly unknown function of the tumor-promoting isoforms, deciphering their functions in tumor biology has an enormous potential to provide insights for the future of cancer diagnosis and therapy.

Tumor-associated isoforms were demonstrated to possess functions that can range from an isoform lacking a regulatory domain, an isoform directly opposing functions of the other isoforms normally expressed in the tissues, and to those with unique functions
discrete from its related isoforms. Their functional diversity was demonstrated to contribute to most hallmarks of cancer. To date, several cancer-associated genes have been reported as aberrantly spliced in cancer cells to express more oncogenic isoform. For example, cyclin D1 is a cell cycle driving proto-oncogene frequently overexpressed in breast cancer. Its activity is tightly regulated by nuclear exclusion and proteolysis following GSK3β-mediated phosphorylation on Threonine 286 (48-49). An alternative splice isoform of cyclin D1, cyclin D1b, lacks a domain containing the T286 phosphorylation site and thus is constitutively localized in the nucleus. Importantly, cyclin D1b drives cell cycle progression and its overexpression is a poor clinical indicator for breast cancer patients (50-51). The Bcl-x is an example of a gene that produces splice isoforms with opposing functions. The long Bcl-x isoform, Bcl-x(L), has anti-apoptotic functions while the short isoform known as Bcl-x(s) acts to promote apoptosis (52-53). An alteration in Bcl-x(L) to Bcl-x(s) isoform ratios in breast cancer has been shown to predict metastasis and disease aggressiveness (54). Whereas alternative splice isoforms of cyclin D1 and Bcl-x carry out related functions, the CD44 splice isoforms appear to serve distinct roles. The CD44 is a ubiquitously expressed cell adhesion molecule that functions as a signaling hub between extracellular matrix and intracellular kinase cascades to carry out different functions. Current evidence suggests that CD44s splice isoform is specifically involved in the epithelial to mesenchymal transition (EMT), cell migration, and tumor cell metastasis. Expression of the CD44s protein is enriched in advanced breast cancers with an aggressive phenotype and metastatic potential (55-56). Aberrant tumor splicing of the genes for cell cycle progression, anti-apoptotic response, and metastasis can upregulate their isoforms that are low abundant in normal cells to
promote these processes. On the other hand, cancer cell-driven alternative splicing can
downregulate isoforms that are well-expressed in the normal tissues, such as a pro-
angiogenic factor, vascular endothelial growth factor A (VEGFA). The VEGFA gene is
alternatively spliced in normal tissues to encode three major isoforms, one of which is
VEGFA-165. However, this isoform is further spliced to produce a protein known as
VEGFA-165b, which differs from VEGFA-165 in only 6 amino acids on the C-terminus
(57). High VEGFA-165b isoform expression was observed in many normal adult tissues
but exclusively abolished by the tumor cells in those same organs (58). Detailed
functional analysis of both isoforms demonstrated that even though VEGFA-165b binds
the VEGF receptors, it blocks co-receptor dimerization and activation necessary for their
pro-angiogenic function (59).

Alternative splicing is an endogenous mechanism tumor cells hijack to alter gene
expression signature and promote biological capabilities during multistep tumor
development. Previous gene microarray platforms failed to detect most alternatively
spliced isoforms but the arrival of quantitative deep RNA sequencing strategies has
opened an access to understanding aberrant splicing in human tumors. A more
comprehensive insight, upstream and downstream of splicing factors, into the aberrant
splicing in breast and other tumor types has potential to improve the stratification of
cancer patients for therapeutic strategies. In addition, numerous pilot studies have
demonstrated a potential use of modified DNA oligonucleotides for effectively reversing
aberrant splicing via specific exon skipping (60-61). Notably, most of the aberrantly
expressed isoforms by the tumor cells are found at low or undetectable level in the adult
tissues making them promising therapeutic targets when the goal is to avoid therapy-
associated side effects. Thus, development of the DNA analogs to target identified
tumor-driving splicing alterations will be the key for a successful implementation of this
therapeutic approach.

I.3. Dmp1 and Senescence

Dmp1 (cyclin D-binding myb-like protein 1), also known as Dmtf1 (cyclin D-binding
myb-like transcription factor 1), was initially identified as a cyclin D2 interacting
protein in a yeast two-hybrid screen (62). Although D-type cyclins were found to
physically interact with Dmp1 within the DNA-binding domain, the physiological role of
this interaction remains unclear (62-63). The Dmp1 protein sequence contains a central
DNA-binding domain and two acidic transactivation domains on the N- and C-termini.
Based on its domain structure, Dmp1 does not fall into any known gene families;
however, it is structurally related to the myb family of proteins as its DNA-binding
domain contains three myb-like repeats. The function of the myb-like repeats in Dmp1
protein also remains unknown but the DNA-binding domain was found to specifically
bind nonameric DNA consensus sequences [CCCG(G/T)ATGT] to activate transcription.
Dmp1’s function as a transcription factor was confirmed when it was discovered that
Dmp1 directly binds to a consensus sequence in the human CD13/aminopeptidase N
promoter and activates its expression (64). However, the activation of CD13 could not
explain a cell cycle arrest induced by Dmp1 thus posing a hypothesis that Dmp1 may
have other transcriptional targets (63). Through an extensive search of Dmp1 binding
sites on endogenous promoters, it was found that the human and murine Arf (p14ARF in
humans; p19Arf in mice) promoters contain high affinity binding sites for Dmp1 (65). Arf
is a tumor suppressor gene encoded by the *Ink4a* locus through an alternative reading frame (66). In the nucleus, Arf protein directly binds Mdm2 and inhibits its ubiquitin ligase activity and thereby increases nuclear accumulation of the p53 tumor suppressor protein (67-69). Through a series of *in vitro* assays, it was demonstrated that the DNA-binding domain of Dmp1 was necessary for activation of *Arf* expression. Overexpression of Dmp1 in wild-type mouse embryonic fibroblasts (MEFs) led to an Arf-dependent accumulation of p53 and cell cycle arrest (65). Thus, a capacity of Dmp1 to potently induce growth arrest of normal cells was dependent on the Arf-p53 pathway.

To delineate endogenous functions of Dmp1, Inoue *et al* established a first *Dmp1* knockout mouse model using the conventional gene targeting methods (70). Upon isolation of MEFs from these animals, it was observed that loss of *Dmp1* dampened the Arf-p53 pathway and increased cell proliferation compared to the MEFs from *Dmp1* proficient mice. Importantly, when *Dmp1*-null MEFs were passaged *in vitro* using a defined 3T3 protocol, the cells failed to achieve replicative senescence previously described for the wild-type MEFs. Continued proliferation of the *Dmp1*-null MEFs, even after 30 passages, was attributed to reduction in the p53 nuclear accumulation (70). Replicative senescence, first described by Leonard Hayflick, is an intrinsic cellular mechanism that limits the number of replicative cycles a normal diploid cell can complete after which it enters a state of permanent growth arrest (71). After reaching the senescent state, cells acquire a unique morphologic appearance and biochemical signature, which have been used for decades to identify such cells. The senescent cells become enlarged and flattened with condensed heterochromatin foci that can be identified with chromatin binding proteins such as heterochromatin protein 1γ (HP1γ) or
di and tri-methylated lysine 9 on histone H3 (H3K9) (72). Even though they reach an irreversible growth arrest, the senescent cells remain metabolically active and secrete factors that promote an inflammatory response (73-74). Complete contribution of the senescence-associated secretory phenotype (SASP) in normal physiology and cancer remains to be elucidated; though, more recent findings suggest that SASP promotes tumor progression (75). The most prominent and widely used marker of senescent cells is the increased β-Galactosidase (β-Gal) activity, a lysosomal enzyme whose function during senescence still remains unclear (76).

One of the critical sensors of replicative senescence is an attrition of telomere length following each cell division (77). After normal cells reach predetermined telomere length, series of sensors induce p53-dependent cellular senescence to prevent catastrophic DNA loss with consecutive replications (78). An observation that Dmp1 protein accumulates as MEFs are passaged suggested that Dmp1 may also participate in sensing the Hayflick phenomenon. Indeed, loss of Dmp1 blocked activation of the Arf-p53 pathway during replicative stress (70). Although most wild-type cells undergo growth arrest after serial passaging, certain clones escape the senescence program by mutating or deleting the p53 gene (79). Indeed, Dmp1-null cells readily escaped replicative senescence but maintained the wild-type p53 even after 30 passages (70). This finding supports a notion that loss of Dmp1 alleviates a selective pressure to inactivate the p53 pathway during replicative senescence. However, the mechanistic link between replicative stress and Dmp1 activation remains unknown and should be investigated in the future.
Aside from replicative stress, induction of cellular senescence is a major tumor suppressive mechanism guarding cells from hyperproliferating signals. In particular, aberrant expression of oncogenes that drives uncontrolled cell proliferation activates a senescence program known as oncogene-induced senescence (OIS). The OIS was first discovered when several research groups attempted to transform wild-type fibroblasts with the oncogenic H-Ras$^{G12V}$ alone. They quickly discovered that human or mouse cells expressing an oncogene like mutant Ras undergo growth arrest and senescence due to the p53 pathway activation (80-82). It appeared that this was an evolutionary conserved failsafe mechanism that prevents transformation of the cells that acquire oncogenic mutations. To bypass the OIS and establish transformed cell lines that proliferated in culture, inactivation of the p53 pathway was a necessary step during the transformation process. A long existing observation that a majority of human cancers inactivate both alleles of $p53$ further supports the paradigm where early stages of oncogenesis are suppressed by the p53-depenent senescence program (83). As an in vivo confirmation of this phenomenon, the oncogenic Ras (K-Ras$^{G12V}$) was expressed in mouse bronchiolo-alveolar epithelial cells of the lung to show low tumorigenic incidence even though the oncogene was activated in a large number of the cells (84). In addition to mouse data, strong evidence of OIS in human tissues was shown in the skin naevi, benign melanocyte lesions expressing the oncogenic BRAF. The cells within the naevi contained normal telomere length, thus strongly suggesting that the observed senescence hallmarks are a result of OIS and not of the replicative senescence (85). Hence, oncogene induced senescence is a genuine physiologic mechanism that prevents tumor progression of mouse and human premalignant lesions.
At the same time that OIS was recognized as a critical safeguarding tumor suppressor mechanism that prevents cancer development, the question of how tumor cells escape OIS in the lesions that have progressed became an important area of investigation. In addition, signaling pathways linking oncogenic stress and activation of the OIS needed to be understood in order to define the OIS evasion mechanisms during a tumorigenic process. Since early studies in Dmp1-null MEFs demonstrated an escape from the replicative senescence, it was postulated that Dmp1 is also part of the OIS signaling cascade. Indeed, Dmp1 was shown to be a critical sensor of oncogenic stress stemming from the mutant Ras to activate the p53 pathway (70). Sreeramaneni et al mapped the Ras-Raf-Mek-Erk-Ap1 signaling pathway that upregulates Dmp1 expression. In support of Dmp1’s role in OIS, Dmp1-null MEFs could be transformed with the mutant Ras alone while maintaining the wild-type p53 (86). Hence, Dmp1 was discovered as a major sensor of oncogenic stress stemming from a constitutively active Ras signaling. In prostate cancer, the major oncogenic event occurs with the inaction of Phosphatase and tensin homolog (Pten) tumor suppressor protein that negatively regulates the action of PI3-Kinase. Loss of Pten in prostate epithelial cells constitutively activates the PI3K-Akt-mTor pathway and drives uncontrolled cell proliferation. However, the oncogenesis of Pten deficient prostate epithelia is initially prevented by the p53-dependent senescence (87). The mechanistic link between a constitutively active PI3K pathway and the p53 response and how Pten-null cancer cells escape the senescence remains to be determined. Similar to the Ras-induced senescence, overexpression of Erbb2 in mammary epithelial cells also activates the p53-dependent senescence program (88). Hyperproliferation as a result of Erbb2 expression induced the ataxia telangiectasia mutated (ATM)-mediated
DNA damage response (DDR), which was responsible for the p53 activation (89). Thus in the case of Erbb2-induced mammary tumorigenesis, DDR is part of a signaling cascade that activates the senescence program. A link between Erbb2-driven proliferative signal and the DDR-induced senescence program still remains uncertain.

The *in vivo* connection between Dmp1 function and senescent response was first observed when the *Dmp1*-null mice were neonatally treated with gamma ionizing radiation (γ-IR) or dimethylbenzantracene (DMBA), both potent inducers of the DDR. The *Dmp1* deficient mice treated with either γ-IR or DMBA were highly susceptible to a variety of tumors within first year of their life when compared to their *Dmp1* proficient littermates (70). Since DDR is a mediator of replicative senescence during telomere attrition, the findings from *Dmp1*-null mice strongly suggested that Dmp1 functionally sits between the DDR signaling and senescence. In the absence of DDR-inducing agents, normal ageing process during a cell’s or an organism’s lifespan accumulates DNA damage, which activates the DDR and senescence to prevent cancer development. Indeed, *Dmp1*-null mice developed spontaneous tumors in their second year of life further reinforcing an important *in vivo* role of Dmp1 during DDR and replicative senescence (90). The first *in vivo* evidence of Dmp1’s involvement in OIS was demonstrated when the *Eμ-Myc* mouse model of B-cell lymphoma was crossed onto a *Dmp1*-null background to observe accelerated tumorigenesis. Another important observation from these experiments was a lack of phenotypic difference between the *Dmp1*+/− and *Dmp1*−/− backgrounds in *Eμ-Myc*-driven tumor susceptibility suggesting that Dmp1 is a haploinsufficient tumor suppressor. Both *Dmp1* genotypes maintained wild-type Arf-p53 pathway and demonstrated that one copy loss of *Dmp1* was sufficient to
alleviate the OIS (90). Predictably, the critical role of Dmp1 in the OIS was also observed in the K-RasLA mouse model of lung cancer since the Dmp1-null mice most commonly developed lung tumors and Dmp1 was critical in blocking the Ras-induced cell transformation. As in the Eμ-Myc-driven lymphomas, Dmp1 loss accelerated lung tumorigenesis without a difference between the Dmp1+/− and Dmp1−/− backgrounds. Notably, K-RasLA lung tumors in the Dmp1+/+ background naturally deleted one Dmp1 allele in ~42% of cases, while the Dmp1 locus was always kept intact in the K-RasLA tumors on the p53-null background. Remaining Dmp1 allele in the naturally occurring Dmp1+/− tumors never displayed promoter hypermethylation or mutations providing an evidence of true haploid insufficiency (91). Hence, aside from confirming that Dmp1 function in the OIS is dependent on p53, these findings also provide a mechanism cancer cells could utilize to inactivate the Dmp1-Arf-p53 tumor suppressor pathway in mouse tumors.

Human DMP1 protein is encoded by a locus mapped to the chromosome 7q21. Structural comparison between the mouse and human Dmp1 proteins shows high similarity with 95% amino acid identity. They share perfect identity between a.a. 125-417 which contains the myb-like repeats and DNA-binding domain. A Northern blot analysis of the DMP1 mRNA in normal human tissues showed its most prominent expression in testis, blood leukocytes, thymus, spleen, and brain (92). In 2003, Tschan et al reported that human DMP1 locus encodes three unique splice isoforms, which they detected during a routine cDNA cloning (93). Thorough analysis of DMP1 exons revealed that alternative splicing of DMP1 occurs at a.a. 237, corresponding to the Exon 10, to include previously unrecognized sequences carrying TAA stop codon. Hence,
inclusion of the new sequences at Exon 10 produces shorter protein isoforms that contain 34 or 48 unique amino acids at the C-terminus. The 760 a.a. long tumor suppressor and an activator of the p53 pathway was named as DMP1α, while two new splice isoforms without any known functions were named as DMP1β and DMP1γ. Due to their shorter protein sequence, both of these isoforms lack the DNA-binding and C-terminal transactivation domains. Previous work has demonstrated necessity of those domains, lacking in DMP1β/γ isoforms, for Dmp1α-mediated activation of the CD13/aminopeptidase N promoter. Indeed, both new isoforms failed to activate the CD13/aminopeptidase N promoter, but DMP1β was able to abrogate DMP1α-mediated activation of the same promoter (93). This finding suggested possible opposing functions of DMP1 isoforms. Switching of isoform ratios to increase DMP1β and decrease DMP1α expression during monocytes differentiation to macrophages further suggested non-overlapping functions between these two isoforms. In fact, DMP1β overexpression in monocytes blocked PMA-induced terminal differentiation to macrophages and promoted continual proliferation. The phenotypic observation from Dmp1-null mice also supported a hypothesis that Dmp1 possesses functions other than p53 activation and tumor suppression. When compared to wild-type littermates, Dmp1-null male and female mice showed significant growth retardation with up to 30% lower body mass at birth, which was sustained into adulthood. Additionally, Dmp1 deficient females were unable to nurse newly born offspring due to poor expansion of mammary luminal cell necessary for milk production (70). These published and unpublished observations suggest that Dmp1 locus participates in growth promotion during certain stages of development.
Therefore, future studies will need to focus on precisely delineating role of the Dmp1 locus and its isoforms in normal and disease physiology.

Localization of DMP1 on 7q21, a chromosomal arm frequently associated with 7q abnormalities in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), indicated that it could be one of the loci affected in these diseases. Indeed, one copy of DMP1 locus (hemizygous deletion) was deleted in all of 9 AML and MDS patient samples analyzed (92). Others have also reported frequent hemizygous deletions on chromosome arm 7q, which was associated with poor patient outcome (94-95). In 2007, Mallakin et al reported the first detailed deletion analysis of DMP1-INK4A/ARF-p53 loci in human lung adenocarcinoma (91). Similarly to the findings in K-RasLA mouse model of lung cancer, the DMP1 locus was hemizygously and narrowly deleted in ~35% of human lung tumors which was found mutually exclusive with deletion of the INK4A/ARF or p53. The genes surrounding the DMP1 locus were found unaffected in ~80% of the cases with DMP1 loss demonstrating the specificity for DMP1 inactivation. Haploinsufficiency of DMP1, as seen in K-RasLA and Eμ-Myc-driven mouse tumors, was recapitulated in human cancers with DMP1 hemizygous deletion cases, which maintained the other DMP1 allele wild-type without promoter hypermethylation. The mutual exclusivity between the DMP1 and ARF-p53 deletion strongly supports a physiological dependence of oncogene-induced ARF-p53 pathway on DMP1 expression in human tissues (91). It also supports a paradigm that inactivation of upstream regulators of p53 alleviates selective pressure in tumor cells to delete the p53 locus. Thus, patients with DMP1 loss in lung cancer may have a favorable clinical outcome because they retain
wild-type p53 that could be activated by other signaling pathways or chemotherapeutic drugs that depend on the p53 activation.

Based on the amino acid sequence, human and mouse Dmp1 protein is predicted to migrate to ~85kDa on a SDS-PAGE gel; however, the apparent M.W. of Dmp1 is always observed around ~125-130kDa. This suggests that Dmp1 protein is extensively modified decreasing its mobility in the gel electrophoresis. Indeed, Dmp1 was directly phosphorylated by the cyclin D/Cdk4 complex in vitro (62). The function of Dmp1 phosphorylation or any other post-translational modifications has not been investigated. In addition, Dmp1 amino acid sequence does not contain a canonical nuclear localization signal (NLS) that facilitates import of transcription factors like Dmp1 into the nucleus. Post-translational modification of transcription factors was shown to play a critical role in their functional regulation and nucleocytoplasmic trafficking (96-97). Therefore, delineating phosphorylation and other post-translational modification of Dmp1 could not only reveal a mechanism of nuclear import but provide a better understanding of Dmp1 regulation in normal and cancer cells.

I.4. Overview

Published data from our lab and other groups suggest that the DMP1 locus is a vital mediator of the p53 pathway action in response to an oncogenic transformation. The mouse models of lymphoma and lung cancer strongly support a tumor suppressive role of the Dmp1-Arf-p53 pathway in preventing tumorigenesis, which is evaded by various means of inactivation of any component of this pathway during tumor progression (90-91). Breast cancer is the most common tumor type in women that
contributes to a significant health care burden in the Western societies (98-100). Multiple targeted agents have been developed to treat appropriate subtypes of breast cancer; however, chemotherapeutic regiments consisting mostly of antracycline and cyclophosphamide based drugs continue to play a significant role in improving patient outcome (101). Numerous studies have confirmed dependence of chemotherapeutic efficacy in breast cancer patients on the $p53$ status (102-103). Indeed, $p53$ deletion or mutation status is different among molecular subtypes (the lowest frequency in Luminal A and the highest in basal-like subtype) of breast cancer suggesting that other factors modify or alleviate proclivity of tumors to inactive the p53 pathway. Therefore, defining regulators of the p53 pathway and the contribution of upstream molecular modifiers of p53 in breast cancer will afford a broader understanding of initiation and progression events that could benefit clinical management of breast cancer patients.

Dmp1 is a *bona fide* tumor suppressor that senses oncogenic stress to induce p53-dependent senescence. It is upregulated by oncogenes such as the mutant Ras to block progression of lung tumors and its loss modifies Arf-p53 inactivation in multiple models of cancer (90-91). To date, Dmp1 involvement in the regulation of mammary epithelium and breast tumorigenesis remains unknown. Overexpression of breast cancer-associated oncogene ERBB2, an activator of the PI3K-AKT and MAPK signaling pathways, induces p53-dependent growth arrest in mammary epithelial cells (88-89). However, the signaling connection between ERBB2 and p53-driven cell cycle arrest has not been elucidated. In addition, current evidence suggests that chromosomal region harboring the $DMP1$ locus is frequently deleted in breast cancer (94-95). Therefore, in this thesis we investigated DMP1 functional role in the p53-dependent OIS using mouse models and
clinical breast cancer samples. We hypothesize that Dmp1 is a sensor of oncogenic stress in the mammary gland and its activity mediates the p53 pathway activation. Furthermore, we propose that during breast tumor initiation and progression the DMP1 locus is altered via gene deletion and/or aberrant splicing to escape the OIS and thereby dampen the p53-mediated tumor suppression.
References


CHAPTER II

Critical roles of DMP1 in HER2/neu-Arf-p53 signaling and breast cancer development

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II.1. Abstract

HER2 overexpression stimulates cell growth in $p53$-mutated cells while it inhibits cell proliferation in those with wild-type $p53$, but the molecular mechanism is unknown. The $Dmp1$ promoter was activated by HER2/neu through the PI3K-Akt-NF-κB pathway, which in turn stimulated $Arf$ transcription. Binding of p65 and p52 subunits of NF-κB was demonstrated to the $Dmp1$ promoter and that of Dmp1 to the $Arf$ promoter upon HER2/neu overexpression. Both Dmp1 and p53 were induced in pre-malignant lesions from MMTV-neu mice and mammary tumorigenesis was significantly accelerated in both $Dmp1^{+/−}$ and $Dmp1^{−/−}$ mice. Selective deletion of $Dmp1$ and/or overexpression of Tbx2/Pokemon was found in $>50\%$ of wild-type HER2/neu carcinomas while the involvement of Arf, Mdm2, or p53 was rare. Tumors from $Dmp1^{+/−}$, $Dmp1^{−/−}$, and wild-type neu mice with hemizygous $Dmp1$ deletion showed significant downregulation of $Arf$ and $p21^{Cip1/WAF1}$, showing $p53$ inactivity and more aggressive phenotypes than tumors without $Dmp1$ deletion. Notably, endogenous h$DMP1$ mRNA decreased when $HER2$ was depleted in human breast cancer cells. Our study demonstrates the pivotal roles of Dmp1 in HER2/neu-$p53$ signaling and breast carcinogenesis.

II.2. Introduction

Breast cancer is one of the largest public health issues in the United States and most of the industrialized world (1-4). Breast cancers that are positive for the estrogen receptor (ER) are usually responsive to adjuvant hormonal therapy with anti-estrogens and/or aromatase inhibitors, and thus have a more favorable prognosis (1). On the other hand, ER-negative breast cancers are often associated with aggressive disease, including
amplification of HER2 or c-Myc oncogenes and mutation of the p53 gene (5). Chemotherapy plus use of the humanized monoclonal antibody to HER2 (trastuzumab) is considered the best treatment for hormone-unresponsive or resistant patients, but the prognosis of such patients is poor (5).

HER2/neu encodes a receptor-type tyrosine kinase that belongs to the EGFR family (5-9). It is overexpressed in ~30% of breast cancer cases, primarily due to gene amplification. HER2/neu overexpression is found in metastatic lesions, and thus is associated with poor prognoses (4-6). Recent studies have stressed the importance of phosphatidylinositol-3′-kinase (PI3K) and serine/threonine kinase Akt/protein kinase B in HER2/neu signaling (10). The PI3K-Akt signaling has also been linked to the induction of NF-κB (11-13). Since transcriptional activation by NF-κB requires its nuclear translocation, degradation of IκB molecules triggered by phosphorylation of serine residues 32/36 by IκB kinases has been considered a key rate-limiting step in NF-κB activation (11). Importantly, both human breast cancer cell lines and clinical specimens often show constitutive activation of NF-κB (14), suggesting oncogenic roles of subsets of NF-κB in breast cancer development.

Dmp1, a cyclin D binding myb-like protein 1 (also called Dmtf1), was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait (15). Dmp1 shows its activity as a tumor suppressor by directly binding to the Arf promoter to activate its gene expression, and thereby induces Arf- and p53-dependent cell cycle arrest (16, 17; for Arf reviews, 18, 19). Dmp1-null cells can easily give rise to immortalized cell lines that retain wild-type p19Arf and functional p53 and are transformed by oncogenic Ras alone, suggesting that the activity of the Arf-53 pathway is
significantly attenuated in Dmp1-deficient cells (20, 21). The murine Dmp1 promoter is efficiently activated by oncogenic Ras and is repressed by mitogenic signals mediated by E2Fs and genotoxic signals by NF-κB (22-24; for review ref. 25). Both Dmp1+/− and Dmp1−/− mice are prone to tumor development when neonatally treated with dimethylbenzanthracene or by ionizing radiation (20, 21). Tumors induced by the Eμ-Myc or K-Ras transgene were greatly accelerated in both Dmp1+/− and Dmp1−/− backgrounds with no differences between groups lacking one or two Dmp1 alleles, suggesting haploid-insufficiency of Dmp1 in tumor suppression (21, 26). Moreover, tumors from Eμ-Myc or K-RasLA mice rarely showed p53 mutation or Arf deletion, indicating that Dmp1 is a physiological regulator of the Arf-p53 pathway in lymphoid and lung epithelial cells (21, 26; for reviews refs. 27, 28).

The hDMP1 gene is located on chromosome 7q21, a region often deleted in human breast cancer and hematopoietic malignancies (29-32). We recently found that loss of heterozygosity of hDMP1 was present in ~35 % of non-small cell lung carcinomas (26). It was reported that HER2 overexpression in p53 wild-type human ovarian carcinoma cell line became apoptotic shortly after transfection, while HER2 expression was associated with cell proliferation in cells with mutated p53 (33). However, the signaling pathway that links HER2 overexpression and activation of p53 has never been demonstrated. Moreover, very little is known about the roles of Dmp1 (or hDMP1) in breast cancer development. The current study was conducted to demonstrate the roles of Dmp1 and Arf in HER2/neu signaling and breast carcinogenesis. We show that both Dmp1 and Arf promoters are selectively activated by HER2/neu and both Dmp1 and p53 proteins are induced in pre-cancerous mammary glands from MMTV-neu mice (34). Of
note, the value of MMTV-LTR driven transgenic mice as models for human breast cancer has recently been reconfirmed since the discovery of MMTV env-, LTR-like sequences in 35-40% of human breast carcinomas (35, 36). We crossed MMTV-neu mice with Dmp1-deficient mice to observe the latency period for tumorigenesis, and have conducted extensive molecular genetic analyses of mammary tumors. We also demonstrate that human DMP1 is regulated by endogenous HER2 overexpressed in breast epithelial cells.

II.3. Materials and Methods

Establishment of Dmp1+/−, Dmp1−/−; MMTV-neu compound mice

Dmp1-heterozygous females were backcrossed to the same FVB/NJ males (Jackson Laboratories, #001800) for more than 8 generations to obtain Dmp1+/− mice with >99% FVB/NJ background overall. One male MMTV-neu (mutant) mouse (Jackson Laboratories, #005038) was crossed with two Dmp1+/− females to obtain Dmp1+/−; MMTV-neu mice. Then Dmp1+/−; MMTV-neu compound transgenic mice were further crossed with Dmp1+/− mice to obtain more than 25 mice with each genetic background. Littermate wild-type mice were used as controls. Mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals.

Real-time PCR

Quantitation of Dmp1, p19Arf, p21Cip1/WAF1, and p16Ink4a mRNAs were conducted by real-time PCR Taqman assay by ABI7500 (Applied Biosystems, Foster City, CA) using β-actin as an internal control (23, 24, 37). For p21Cip1/WAF1, Mm01303209_m1 was used; other assays were custom-designed at ABI. Gene copy number assays for Dmp1,
Arf, p53, grm3, and abcb1 were also performed by real-time PCR using β-actin as an internal control (26).

**Western blotting**

Proteins were extracted with ice-cold EBC buffer (15) with proteinase inhibitors from frozen mammary tumor cells or human breast cancer cell lines. After gel electrophoresis and transfer to nitrocellulose membranes, proteins were visualized by immunoblotting with affinity-purified polyclonal antibodies to Dmp1 (RAX) (23), p53 (sc-6243G, Santa Cruz Biotech), Mdm2 (ab16896 [2A10], Abcam), p19Arf (sc-32748), p14ARF (ab3642, Abcam), p16Ink4a (sc-1207), p21CIP1/WAF1 (sc-397G), TBX2 (sc-17880), Pokemon (A300-548A, Bethyl Inc., TX), Twist (sc-15393), or β-Actin (sc-1615, sc-47778), followed by incubation of the filters with HRP-conjugated second antibodies, and reaction with the enhanced ECL detection kit (PerkinElmer).

Cell culture, reporter assays, chromatin immunoprecipitation, *in vitro* mutagenesis of the Dmp1 promoter, immunohistochemical staining, retroviruses for HER2 shRNA, and statistical analyses are described in the Supplementary Materials and Methods.

**II.4. Supplementary Materials**

*Cell culture and reporter assays*

Wild-type and Dmp1-null MEFs were established from 13.5-day-old embryos and maintained as previously described (20). Human breast carcinoma cell lines were obtained from the institutional core facility or purchased from the American Type Culture Collection. For reporter assays, 2 x 10⁵ cells were seeded into 60 mm diameter culture
dishes 24 hours before transfection. In order to study the responsiveness of the Dmp1 and Arf promoters to HER2, 4 μg of luciferase reporter DNA was co-transfected with 0.5 to 1 μg of HER2 expression vector (pME18S-c-ErbB2, gift from Dr. T. Yamamoto, University of Tokyo) and 4 μg of internal control human β-Actin promoter-secreted endocrine alkaline phosphatase vector (a gift from M. Ostrowski, Ohio State University). The luciferase construct for the human p14ARF promoter was obtained from Dr. P. Jones (Univ. of Southern California), the human p27KIP1 promoter was received from Dr. A.T. Look (Dana Farber Cancer Institute), and the murine p16Ink4a promoter (17) was received from Drs. C. Sherr and M. Roussel (St. Jude Children’s Research Hospital). The 1.4 kb genomic DNA for the human Mdm2 (Hdm2) promoter containing both P1 and P2 was cloned in our laboratory. Genejuice (Novagen) was used in all transfections. Specific MAP kinase inhibitors, U0126 (for MEK/ERK), LY294002 (for PI3K), and Akt inhibitors IV and V were purchased from EMD Chemicals (Gibbstown, NJ) and were used at the concentration of 5 μM for U0126 and LY294002, 2.2 μM for Akt inhibitor IV, and 8.3 μM for Akt inhibitor V. PS341 (Velcade™) was purchased from Millennium Pharmaceuticals (Cambridge, MA) and was used at 100 nM. The expression vector for IκBα super repressor was received from Dr. A. Thorburn (Univ. of Colorado).

Chromatin immunoprecipitation

Chromatin immunoprecipitations was performed as described previously (22, 24) (see http://genomecenter.ucdavis.edu/farnham/farnham/protocols/tissues.html for tissue ChIP). The lysates were precipitated with specific antibodies to NF-κB family proteins (all from Santa Cruz Biotech; p50: sc-7178x, p52: sc-7386x, p65: sc-372x, RelB: sc-226x, and c-Rel: sc-71x), or with anti-Dmp1 antibody (RAX or RAD) (23, 37). For
detection of the endogenous NF-κB family transcription factors on the murine Dmp1 promoter, sense primer 5'-AAAGCGAGGTCACACTCACG-3' and antisense primer 5'-CCCGACGTCACACTTCCGTCT-3' were used (shown as SE#8 and AS#8 in Supplementary Fig. S1A). The primers for detection of the endogenous Dmp1 binding to the Arf promoter have been reported (22).

In vitro mutagenesis

The murine Dmp1 promoter deletion/point mutants were generated by use of an in vitro mutagenesis kit (Stratagene). The oligonucleotide sequences used for in vitro mutagenesis of the Dmp1 promoter for the NF-κB site #1 was reported (24). For mutagenesis of the NF-κB site #2, 5'-GCGTGCCGACGCAGACACGCGTGAAGTCGGGGGTGGCGCGC-3' and its reverse complementary sequence were used. The mutated NF-κB site is underlined. The murine Arf promoter construct with a mutant Dmp1/Ets site has been described (17).

Immunohistochemical staining

Immunohistochemical staining of normal and mammary tumor tissues were conducted as described previously (23, 26). These antibodies were used for immunohistochemistry with formalin-fixed, paraffin-embedded sections: Dmp1 (RAX to amino acids 136 to 150 of Dmp1, RAZ to the amino acids 740-756, and RAD to the full length His-Dmp1) (23, 26, 28, 4, 37), p53 (P4235, Sigma), p21Cip1/WAF1 (sc-6246), HER2 (sc-284), ERα (sc-543), PR (sc-538), and Ki67 (SP6, NeoMarkers).

Statistical analyses
Statistical differences of survival in $Dmp1^{+/+}$, $Dmp1^{+-}$, and $Dmp1^{-/-}$; MMTV-neu mice were analyzed by XLSTAT-Life software (Addinsoft). Mann-Whitney test (two-sided) were used to generate the $P$ values (significance level, $\alpha = 0.05$). Statistical analyses of gene expression and histopathology of neu-induced mammary tumors were conducted by two-sided Chi square tests and unpaired Student’s $t$-tests.

**Human breast cancer cell lines**

Human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA).

**Retroviruses for HER2 shRNA**

Amphotropic retroviruses to knockdown HER2 were prepared by using the pSUPERretro RNA interference system (Oligoengine, Seattle, WA). The 19-bp target sequences for HER2 was 5'-GGGGCTGGCTCCGATGTAT-3’ for #3386, 5’-TGGGGTCGTCAAAGACGTT-3’ for #3682 (39). The effectiveness of downregulation of HER2 by shRNA was confirmed by real-time PCR and Western blotting with specific antibodies.

**II.5. Results**

*Both the Dmp1 and Arf promoters are specifically activated by HER2/neu*

We tested whether murine $Dmp1$ and $Arf$ promoter can be activated by overexpression of HER2 (Fig. S1A). Both promoters were activated by HER2 expression in dose-dependent fashion (Fig. 1A, left panel). Both human $DMP1$ and $p14^{ARF}$ promoters were also responsive to HER2 (Supplementary Fig. S1B). The specificity of
these promoter activations by HER2 was confirmed by reporter assays with p27^{KIP1}, p16^{Ink4a}, and Hdm2 promoters, which were all repressed by HER2 (Supplementary Fig. S1B). We then confirmed the results of reporter assays by quantitating the endogenous Dmp1 and Arf mRNA by real-time PCR in wild-type MEFs infected with HER2 virus (Fig. 1A, middle). The Dmp1 protein induction by HER2 was confirmed by transiently expressing HER2 in 3T3 cells (Fig. 1A, right). The Dmp1 promoter activation by HER2 was not inhibited by the MEK-ERK inhibitor, but was completely inhibited by the PI3K inhibitor and by the Akt inhibitors (Fig. 1B). The Dmp1 promoter was efficiently inhibited by proteosomal NF-κB inhibitor PS341, or by co-expression of a constitutively-active IκB super repressor (Fig. 1B). Consistent with these findings, in vitro mutagenesis of either NF-κB site #1 or #2 significantly decreased the responsiveness of the Dmp1 promoter to HER2 (Fig. 1C). We confirmed significant binding of the endogenous p65/relA and p52 subunits of NF-κB to the Dmp1 promoter upon HER2 overexpression by chromatin immunoprecipitation (ChIP) (Fig. 1D, left). Binding of p65 and p52 to the Dmp1 promoter was also confirmed by tissue ChIP with lysates from MMTV-neu tumors (Fig. 1D, right).

We then mapped the HER2-responsive element on the Arf promoter. When the Dmp1/Ets site was mutated, the Arf promoter was not responsive to HER2 expression, suggesting that the promoter activation was Dmp1/Ets-dependent (Fig. 2A, middle). The Arf promoter activation by HER2 was dependent on Dmp1 since the promoter was not activated in Dmp1^{-/-} cells (Fig. 2A, right). Binding of endogenous Dmp1 to the endogenous Arf promoter was confirmed by ChIP with lysates from 4 independent neu
tumors (Fig. 2B). Thus, our data indicate that HER2/neu stimulates the Arf-p53 pathway through activation of the novel PI3K-Akt-NF-κB-Dmp1 signaling (Fig. 2C).

**Acceleration of neu-induced mammary tumor development in Dmp1-knockout mice**

**MMTV-neu** females develop multiple mammary tumors (~5 mm in diameter) with a mean latency of 7 months in FVB/N strain. Since the Dmp1 promoter is selectively activated by HER2/neu, we studied if Dmp1 and p53 proteins were induced in response to active neu in pre-malignant lesions (i.e. hyperplastic, non-transformed mammary glands mixed with islands of early stage tumors) found from 5.5 months old MMTV-neu females. Real-time PCR analysis showed upregulation of the Dmp1 mRNA in early stage mammary tumors (Fig. 3A, left panel). Significant induction of the Dmp1 protein in hyperplastic pre-malignant lesion was confirmed by immunohistochemical staining of mammary glands adjacent to neu tumors (Fig. 3A, right panel, arrows, n=5, P < 0.001). The p53 protein was barely detectable in normal mammary glands (Fig. 3B, left), but significant amount of p53 was induced in hyperplastic mammary glands from neu mice (Fig. 3B, middle, arrow, P < 0.001). We also observed significant induction of p21^{Cip1/WAF1} in early stage neu tumors (Fig. 3B, right, arrow, P < 0.001). On the other hand, the pro-apoptotic p53 target, Puma was not induced in early stage neu tumors (data not shown). Our data indicate activation of the Dmp1-p53-p21^{Cip1/WAF1} signaling in pre-malignant hyperplastic mammary glands or early stage mammary tumors in response to oncogenic HER2/neu signaling.

To study the cooperation between Dmp1-loss and HER2/neu overexpression/activation in vivo, we crossed MMTV-neu mice with Dmp1-null mice. Mammary glands from Dmp1^{−/−} or Dmp1^{+/−} virgin females are morphologically
indistinguishable from those from wild-type females, with nearly the same staining patterns for ER, PR, and Ki67 (Supplemental Figures S2 and S3). HER2/neu-induced mammary tumor development was significantly accelerated from 200 days to 162 days in $Dmp1^{+/\cdot}$ and 154 days in $Dmp1^{-/-}$ mice ($P < 0.0001$), with no statistically significant differences between $Dmp1^{+/\cdot}$ and $Dmp1^{-/-}$ (Fig. 3C). Analysis of genomic DNA from mammary tumors of $Dmp1^{+/\cdot}$ mice showed that the wild-type $Dmp1$ locus was retained in all the eight tumors examined (Fig. 3C). The tumors from $Dmp1^{+/\cdot}$ mice expressed the Dmp1 protein, showing haploid insufficiency (Fig. 3D). p53 was barely detectable in the mammary glands or tumors from $Dmp1^{+/\cdot}$ or $Dmp1^{-/-}$ mice, suggesting inactivity of the p53 pathway (Fig. 3D). These data indicate that Dmp1 has a critical role both as a mediator of HER2-p53 signaling and in prevention of neu-induced mammary tumor development.

**Frequent deletion of Dmp1 in neu-induced mammary tumors**

The Dmp1 protein was often downregulated in tumor tissues in comparison to pre-malignant mammary glands in wild-type MMTV-neu mice (Fig. 3A). To investigate the molecular mechanism for this finding, we studied the gene copy numbers of Dmp1 by real-time PCR (Fig. 4A). One allele of the Dmp1 gene was deleted in 6 of 10 mammary tumors from single MMTV-neu transgenic mice, and 6 of 8 tumors in double neu transgenic mice (Fig. 4A). On the other hand, one allele of Arf was lost only in 1 of 10 tumors, and none of the tumors showed $p53$ deletion (Supplementary Fig. S2). Specific deletion of Dmp1 was further confirmed by real-time PCR analyses of grm3 and mdr1 (Fig. 4B). The tumors with hemizygous deletion for Dmp1 ($Dmp1^{wt}$ HD) expressed significantly lower levels of Dmp1 mRNA than those without Dmp1 deletion ($Dmp1^{wt}$...
ND) \( (P < 0.0001, \text{Fig. 4C}) \). The \( Dmp1 \) mRNA was significantly downregulated in tumors from \( Dmp1^{+/+} \) mice than in \( Dmp1^{wt\text{ND}} \) \( (P < 0.0001) \), but the levels were not different from those of \( Dmp1^{wt\text{HD}} \) tumors (Fig. 4C). \( p19^{Arf} \) mRNA expression was lower in mammary tumors from \( Dmp1^{wt\text{HD}}, Dmp1^{+/+}, \) and \( Dmp1^{-/-} \) mice than in \( Dmp1^{wt\text{ND}} \) tumors, but the levels were not significantly different among tumors from \( Dmp1^{wt\text{HD}}, Dmp1^{+/+}, \) and \( Dmp1^{-/-} \) mice (Fig. 4D). Likewise, the p53 target, \( p21^{Cip1/WAF1} \) mRNA expression was significantly downregulated in mammary tumors from \( Dmp1^{wt\text{HD}}, Dmp1^{+/+}, \) and \( Dmp1^{-/-} \) mice in comparison to \( Dmp1^{wt\text{ND}} \) (Supplementary Fig. S3A) while the expression of pro-apoptotic target \( Puma \) did not change significantly among the three \( Dmp1 \) genotypes (data not shown). The \( p16^{Ink4a} \) mRNA expression in tumors from \( Dmp1^{wt\text{HD}} \) and \( Dmp1^{+/+} \) tumors was not significantly different from that in \( Dmp1^{wt\text{ND}} \), while it was downregulated in \( Dmp1^{-/-} \) (Supplementary Fig. S3B).

We then studied protein expression involved in the Arf-Mdm2-p53 tumor surveillance pathway and \( Ink4a/Arf \) modulators in \textit{neu} tumors from the three \( Dmp1 \) genetic backgrounds. The \( Dmp1 \) protein expression was 2-10 times higher in wild-type \textit{neu} tumors than in non-transgenic wild-type mammary glands (MMG) from 12-week-old virgin females (Fig. 5A), reflecting the promoter activation. The \( Dmp1 \) levels were higher in \( Dmp1^{wt\text{ND}} \) tumors than in \( Dmp1^{wt\text{HD}} \) tumors. The \( Dmp1 \) protein expression was higher than that in normal mammary glands in some \( Dmp1^{+/+}; \textit{neu} \) tumors or at the levels of normal mammary glands in others (Fig. 5B). None of the tumors from the three \( Dmp1 \) genotypes overexpressed \( p19^{Arf} \) or p53 at the level of the \( p53^{mutant} \) cell line (Fig. 5), suggesting that \textit{neu} tumors of the three different \( Dmp1 \) genotypes retained wild-type p53. Sequencing of the \( p53 \) cDNAs confirmed that these tumors expressed wild-type p53.
regardless of the *Dmp1* genotype. Mdm2 was not overexpressed in any of the *neu* tumors (Fig. 5). Tbx2 overexpression was found in ~70% of the tumors from *Dmp1* wild-type mice and in 20-30% of *Dmp1*+/− and *Dmp1*−/− tumors (Fig. 5). Pokemon overexpression was found in nearly all the tumors from HER2/neu tumors, regardless of the *Dmp1* genotype, while none of the mammary tumors overexpressed Twist (Fig. 5). None of the MMTV- *neu* tumors overexpressed Tbx3 or Bmi1 (data not shown). Together, our molecular genetic analyses of *neu*-induced mouse mammary tumors showed that 1) hemizygous deletion of *Dmp1* is found in ~50% of wild-type *neu* tumors, 2) mutation/deletion/overexpression of key components of the Arf-Mdm2-p53 pathway is rare in *neu* tumors, 3) both *p19Arf* and *p21Cip1/WAF1* mRNAs are significantly downregulated in *Dmp1*wt HD, *Dmp1*+/+, and *Dmp1*−/− tumors in comparison to *Dmp1*wt ND tumors, showing the inactivity of the p53 pathway and the mechanism of haploid insufficiency of Dmp1, 4) *p16Ink4a* mRNA level is low only in *Dmp1*−/− mammary tumors, and that 5) both Tbx2 and Pokemon proteins are often overexpressed in wild-type *neu* tumors; Tbx2 overexpression is less frequent in *Dmp1*+/− or *Dmp1*−/− tumors while Pokemon overexpression is independent of the *Dmp1* genotype.

*Histopathological features of HER2/neu tumors from Dmp1-deficient mice*

Macroscopically tumors from *Dmp1*+/− or *Dmp1*−/− mice showed more aggressive phenotypes (i.e. high nuclear grade, local invasion, increased angiogenesis and metastasis) than those from *Dmp1*+/+ mice. At sacrifice, the total tumor weight was significantly increased in *Dmp1*+/− (P = 0.039) and *Dmp1*−/− (P = 0.0015) mice compared to *Dmp1*+/+ mice (Fig. 6A, B). Metastatic disease was more frequent in *Dmp1*−/− (4/26, 15.4%) or *Dmp1*+/− mice (4/37, 10.8%) than in *Dmp1*+/+ mice (2/35, 5.7%). We
therefore categorized tumors from MMTV-neu mice using the published criteria (38). Grade A is low grade nodular mammary tumor with uniform nuclear size with low mitotic count (Fig. 6C, left upper panel, found in Dmp1+/+); grade B is intermediate, invasive acinar mammary adenocarcinoma with small and large nests of cancer cells infiltrating the mammary stroma (right upper and left lower panels, from Dmp1+/−); grade C is a high-grade, solid, invasive carcinoma with remarkably high mitotic figure count and central comedo necrosis with high-grade nuclei (right lower panel, from Dmp1−/−) (38). Tumors from both Dmp1+/− and Dmp1−/− mice showed significantly increased scores of grade B and C tumors, showing the more aggressive and invasive nature of tumor development, while there was no significant difference in the features between one allele and two alleles loss of Dmp1 (Fig. 6D, upper panel). When Dmp1 wild-type tumors were compared between HD and ND groups, tumors with Dmp1 deletion showed significantly increased scores for grade B (P = 0.0033), but not for grade C (Fig. 6D, lower panel). The difference in the invasiveness between Dmp1wt HD tumors and Dmp1+/− tumors can be explained by the duration of Dmp1 deletion, which should be significantly longer in tumors from Dmp1−/− mice. Thus, our data indicate that loss of Dmp1 contributes to the more invasive and metastatic phenotypes of mammary carcinomas in neu-transgenic mice.

*Endogenous HER2 upregulates hDMP1 mRNA in human breast epithelial cells*

To study whether the endogenous HER2 upregulates hDMP1 in human breast epithelial cells, hDMP1 levels were quantitated by real-time PCR. We found that hDMP1 mRNA levels were significantly higher in human breast cancer cell lines with HER2 overexpression than those with low or no HER2 expression (P = 0.0076,
Supplementary Fig. S6A). Downregulation of endogenous HER2 with two different shRNAs (39) (> 95 %) resulted in significant decrease of the hDMP1 mRNA in three different HER2-amplified human breast cancer cell lines, SK-BR-3, BT-474, and HCC1569 (Supplementary Fig. S6B, HCC1569 data not shown). The p14\textsuperscript{ARF} mRNA also decreased in BT-474 cells treated with shRNA to HER2 (data not shown). Inhibition of PI3K, Akt, or NF-κB activity by specific inhibitors downregulated endogenous hDMP1 levels in these breast cancer cells (Supplementary Fig. S6C), and induced cell cycle arrest or apoptosis in SK-BR-3 and BT-474 cells (Supplementary Fig. S6D). Thus, overexpression of HER2 increases endogenous hDMP1 through activation of the PI3K-Akt-NF-κB pathway.

II.6. Discussion

In this study, we have characterized the signaling pathway that links HER2/neu overexpression and p53 activation. Although HER2 overexpression activates both Ras-Raf-MEK-ERK-AP1 and PI3K-Akt-NF-κB signaling, our study shows that HER2-Dmp1 signaling is independent of the former signaling cascade. Dependence of the Dmp1 promoter activation by NF-κB was confirmed by 1) proteasomal inhibitor PS341 treatment, 2) expression of IκBα super repressor, and by 3) mutating the NF-κB sites on the Dmp1 promoter. Moreover, we could confirm the binding of endogenous p65 and p52 subunits of NF-κB to the endogenous Dmp1 promoter in HER2 virus-infected cells as well as in mammary tumors from MMTV-neu mice by tissue ChIP. It has been reported that phosphorylation of p65 Ser536 in transactivation domain 1 by IKK or other kinases can stimulate p65 transactivation (40). On the other hand, phosphorylation of
p65 Thr-505 in transactivation domain 2 by Chk1 results in transcriptional repression of some NF-κB target genes, by increased association of p65 with HDAC1 (41,42). Phosphorylation of p65 at Thr-505 occurs when the cells are exposed to genotoxic stimuli. Thus, NF-κB plays roles in both activation (HER2/neu, this study) and repression (genotoxic stimuli) (24) of the Dmp1 promoter dependent on the stress the cells receive.

It was reported that p19Arf inhibits HER2/neu-mediated oncogenic growth by antagonizing Akt-mediated p27Kip1 phosphorylation and increasing p27Kip1 stability (43). Our study showed that the Arf promoter is activated by HER2/neu. Wild-type MMTV-neu tumors that retained two alleles of Dmp1 expressed the Arf mRNA at levels 2-40 times higher than that in normal mammary epithelial cells. Induction of p19Arf by HER2/neu is largely dependent on Dmp1 since 1) the Arf promoter activation was not found in Dmp1-deficient cells, and 2) Arf mRNA levels were significantly lower in Dmp1−/−, Dmp1−/+c, and in Dmp1wtf HD; neu tumors than in Dmp1wtf ND. The critical role of the Dmp1-Arf-p53 pathway in preventing mammary tumorigenesis was demonstrated by immunohistochemical staining of pre-neoplastic regions found in early stage neu tumors, where we found significant upregulation of Dmp1, p53, and p21Cip1/WAF1 proteins. Although significant induction of Arf mRNA was detectable by real-time PCR in Dmp1wtf ND; neu tumors, immunohistochemical demonstration of p19Arf in pre-malignant mammary tissue was technically difficult possibly because the absolute expression levels of p19Arf were very low. It is generally believed that very low levels of Arf are enough to show tumor suppressive activity, and that further induction provides the selective pressure for the emergence of tumors that have inactivated the gene (44).
Interestingly, the mouse Dmp1 gene was hemizygously deleted in ~50% of neu mammary tumors with significant downregulation of the Dmp1 protein. The gene deletion was limited to the Dmp1 locus in 80% of the mouse tumors, according to our analysis of neighbor gene deletions by real-time PCR. However, neu-induced mammary tumors are different from human breast cancers in that the Ink4a/Arf or p53 locus is not frequently involved. Mdm2 overexpression was not observed in any of the neu tumors, regardless of the Dmp1 genotype. In contrast, we found frequent overexpression of Ink4a/Arf repressors, Tbx2 and Pokemon, in HER2/neu tumors. The frequency of Pokemon overexpression did not change significantly in Dmp1+/− and Dmp1−/− tumors, while the frequency of Tbx2 overexpression was decreased from 70% to 20-30% in Dmp1+/− and Dmp1−/− tumors. This indicates that Dmp1 deletion may alleviate the function of Tbx2 overexpression to some extent. It has been reported that TBX2 is amplified in 8.6-21.6% of sporadic human breast carcinomas (45), and ectopic expression of Tbx2 results in DNA polyploidy and cisplatin resistance (46). On the other hand, very little is known about the role of Pokemon in human breast cancer (47). Further studies will be required to reveal how these Ink4a/Arf repressors collaborate with Dmp1-loss in breast (or mammary) carcinoma development.

Our current study clearly demonstrates the haploid insufficiency of Dmp1 in neu-induced mammary tumor suppression. Consistent with these findings, the p19^{hrf} and p21^{Cip1/WAF1} mRNA levels were significantly downregulated in both Dmp1+/− and Dmp1−/− tumors, with no differences between the two cohorts. Of note, downregulation of Arf and p21^{Cip1/WAF1} was also observed in Dmp1^{wt} HD tumors, indicating that naturally occurring hemizygous deletion of Dmp1 inactivates the Arf-p53 pathway as well. In either case,
loss of *Dmp1* was associated with more aggressive disease than *Dmp1*<sup>wt</sup> ND tumors. In contrast, *p16<sub>Ink4a</sub>* mRNA was downregulated only in mammary tumors from *Dmp1<sup>−/−</sup>* mice as compared to *Dmp1*<sup>wt</sup> ND mice, suggesting differential regulation of the *Ink4a* and *Arf* promoters by Dmp1. This can be explained by the fact that the *p16<sub>Ink4a</sub>* promoter lacks typical Dmp1 consensus sequences, at least within 500 bps from the transcription initiation site (17, 48).

In conclusion, our study demonstrates the novel signaling cascade that links HER2/neu and p53. Since Dmp1 is induced in pre-malignant tissues, activation of Dmp1 by small molecules may be a reasonable approach to prevent breast cancer development.

**II.7. Acknowledgements**

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References


Figure 1. Both the Dmp1 and Arf promoters are specifically activated by HER2/neu. 

A, left panel. Murine Dmp1 (-374 NsiI) (22) and Arf promoter (-281 BamHI) (17) luciferase constructs were co-transfected with increasing amounts of HER2 expression vector in MCF10A cells. A, middle panel. Wild-type MEFs were infected with a retrovirus expressing HER2 and mRNA for Dmp1 and Arf were quantitated by real-time PCR Taqman assay. A, right panel. NIH 3T3 cells were transfected with HER2 expression vectors and the Dmp1 protein was analyzed with specific antibody. B, the Dmp1 promoter activation by HER2 is inhibited by LY294002 (PI3K inhibitor), Akt inhibitor IV/V, PS341 (proteosomal NF-κB inhibitor), and IκBα SR (super repressor) demonstrating critical involvement of the PI3K-Akt-NF-κB pathway. C, the Dmp1 promoter activation by HER2 is dependent on the integrity of the two NF-κB sites. Mutation of the NF-κB site #1 caused partial inhibition while that of NF-κB site #2 nullified the effects of HER2 on Dmp1 transcription. D, left panel. Chromatin immunoprecipitation (ChIP) analysis of NF-κB binding to the Dmp1 promoter on HER2 expression. Binding of endogenous NF-κB to the Dmp1 promoter was observed with HER2 virus infection of NIH 3T3 cells. D, right panel. Binding of p65 and p52 to the endogenous Dmp1 promoter in mammary tumors (T1 and T2) from MMTV-neu mice.
Figure 2. Activation of the Arf promoter by HER2 and the signaling pathways that link HER2 and p53.

A, activation of the Arf promoter by HER2 is dependent on Dmp1. Reporter assays were conducted in 3T3 cells (left and middle panels) or in Dmp1−/− cells (right panel). The baseline (without HER2 expression vector) was set at 1.0. B, binding of endogenous Dmp1 to the Arf promoter in four different MMTV-neu tumors. Tissue ChIP was conducted with formalin-fixed tumors from wild-type MMTV-neu mice. The Dmp1 protein was detectable on the Arf promoter with two different antibodies to Dmp1 (RAX for T1 and T2, and RAD for T3 and T4). C, proposed signaling pathway that links HER2/neu overexpression and p53 activation. Activation of the Dmp1 promoter is mediated by PI3K-Akt-NF-κB signaling, and induction of Arf by HER2/neu is dependent on Dmp1. This diverts toxic hyperproliferative signaling from HER2/neu to a p53-dependent cell cycle arrest or apoptosis.
Figure 3. Induction of Dmp1 and p53 in vivo by HER2/neu and acceleration of neu-induced mammary carcinogenesis in Dmp1-knockout mice.

A, left. Real-time PCR analysis of the Dmp1 mRNA in early stage mammary tumors from MMTV-neu mice (5-6 months old, 1-2 mm tumors, n = 3). A, right. Detection of the Dmp1 protein in hyperplastic, but non-transformed mammary glands adjacent to a neu tumor (arrows). Note that once a mouse develop mammary tumor (T), the Dmp1 expression levels go down due to hemizygous gene deletion (Fig. 4A). B, left. p53 is barely detectable in normal mammary glands (arrows). B, middle. Induction of the p53 protein in hyperproliferative mammary glands (arrow) from a MMTV-neu mouse. p53 is significantly downregulated in the tumor (T). B, right. Induction of the p21Cip1/WAF1 protein in early stage mammary tumor (arrow) from a MMTV-neu mouse. p21Cip1/WAF1 is hardly detectable in the tumor (T). C, left. Tumor-free survival of Dmp1+/+ (blue), Dmp1+/− (pink), and Dmp1−/− (green); MMTV-neu compound transgenic mice. Tumor development was significantly accelerated on Dmp1−/− and Dmp1+/− genetic backgrounds as compared to wild-type mice (both P < 0.0001). C, right. Retention of the wild-type Dmp1 locus in mammary carcinomas from Dmp1+/−; neu mice. D, left. Detection of the Dmp1 protein in Dmp1+/−; neu tumor and neighboring tissue. Right. Background signals from a mammary carcinoma from a Dmp1+/−; neu mouse. D, right. p53 is barely detectable in neu-induced mammary tumors from a Dmp1+/− (left) or a Dmp1−/− mouse (right). Scale bars are 100 μm.
Figure 4. Specific deletion of Dmp1 and expression of Dmp1/Arf mRNA in neu-induced mammary tumors.

A, real-time PCR analysis of the Dmp1 copy numbers in wild-type neu tumors showing Dmp1 deletion in 60% of single neu-transgenic and 75% of double neu-transgenic mice. B, the grm3 (glutamate receptor 3) gene, which is located ~500 kb upstream from the Dmp1 locus, was not deleted in any of the neu tumors. The mdr1 (multi drug resistance 1) gene, located ~500 kb downstream from the Dmp1 locus, was deleted in only 2 of 10 cases examined. C, relative expression of the Dmp1 mRNA in mammary carcinomas from MMTV-neu mice. Dmp1^WT ND indicates tumor cells without Dmp1 deletion; Dmp1^WT HD (red) shows those with hemizygous deletion of Dmp1. D, relative expression of the Arf mRNA in mammary carcinomas from neu mice. The Arf expression was significantly downregulated in Dmp1^WT HD, Dmp1^+/-, and Dmp1^-/- tumors.
Figure 5. Analyses the Arf-Mdm2-p53 pathway and Ink4a/Arf modulators in HER2/neu tumors.

A, analyses of wild-type MMTV-neu mammary tumors. The Dmp1 protein expression was 2-16 folds higher in tumors from Dmp1 wild-type mice than normal mammary glands (Dmp1+/+ MMG from 15-week-old non-lactating females), reflecting the Dmp1 promoter activation by HER2/neu. None of the tumors showed p53 mutation or Mdm2 overexpression. The Arf gene was not deleted in any of the mammary tumors. As a positive control, lysates of NIH 3T3 cells were used for Dmp1, Tbx2, Pokemon, and Twist; immortalized MEF with p53 mutation was used for p19Arf and p53; and dm3T3 cells were used for Mdm2. B & C, analyses of Dmp1+/− and Dmp1−/−; neu mammary tumors. p19Arf or p53 was barely detectable in mammary tumors from Dmp1+/− and Dmp1−/− mice. The frequency of Tbx2 overexpression was significantly lower in these tumors than those from wild-type MMTV-neu mice while the pattern of Pokemon overexpression did not change in Dmp1-knockout tumors. MMG: mammary glands.
Figure 6. Histological grading of neu-induced mammary tumors dependent on the Dmp1 genotype.

A, total tumor weight per mouse (mean +/- SD). Dmp1\textsuperscript{+/−} and Dmp1\textsuperscript{−/−} tumors were significantly heavier, showing accelerated growth. B, pictures of mammary tumors found in Dmp1\textsuperscript{+/+} (left upper panel), Dmp1\textsuperscript{+/−} (right upper panel), and Dmp1\textsuperscript{−/−} (lower panels) neu mice. Arrows show the location of tumors. C, mammary tumors from neu transgenic mice were classified into grades A to C (38). Scale bar in C is 100 μm. D, upper panel. Grading of mammary carcinomas from neu mice dependent on the Dmp1 genetic background. D, lower panel. Differential grading of wild-type neu tumors by deletion of Dmp1.
Supplementary Figure S1. Potential transcription factor-binding sites on the murine Dmp1 promoter and responsiveness of the hDMP1, p14\textsuperscript{ARF}, p27\textsuperscript{KIP1}, p16\textsuperscript{Ink4a}, and Hdm2 promoters to HER2.

\textbf{A}, potential transcription factor binding sites on the murine Dmp1 promoter. The Dmp1 promoter has an AP-1 site responsible for oncogenic Ras (22), E2F sites for cell cycle dependent expression of Dmp1 (23), and NF-κB sites for Dmp1 repression by genotoxic stimuli (24). The two NF-κB consensus sequences on the promoter are shown in red. The transcription initiation site is shown as “G” in bold. 

\textbf{B}, effects of HER2/neu overexpression on hDMP1, p14\textsuperscript{ARF}, p27\textsuperscript{KIP1}, p16\textsuperscript{Ink4a}, and Hdm2 promoters. Reporter assay was performed in MCF10A cells with increasing amount of the HER2/neu expression vector. The hDMP1 promoter was efficiently activated while other promoters were repressed by HER2/neu overexpression in mammary epithelial cells.
Supplementary Figure S2. Analysis of mammary glands from Dmp1-knockout mice.

Mammary glands were isolated from twelve-week-old virgin females of each genotype, and were studied by whole mount (upper panels) and by H&E staining (lower panels). Mammary gland branching was slightly underdeveloped in $Dmp1^{-/-}$ tissue, but was histologically normal by H&E staining. Scale bar is 100 μm.
Supplementary Figure S3. Immunohistochemical analysis of mammary glands from Dmp1-knockout mice.

Mammary glands were isolated from twelve-week-old virgin females of each Dmp1 genotype, and were stained for estrogen receptor (ER, sc-543), progesterone receptor (PR, sc-538), or Ki67 (SP6, NeoMarkers). There was no significant difference in the staining pattern for these proteins among the three Dmp1 genotypes. Scale bar is 100 μm.
Supplementary Figure S4. The \textit{p19Arf} and \textit{p53} genes are not frequently deleted in wild-type MMTV-\textit{neu} mammary tumors.

The copy numbers for \textit{p19Arf} and \textit{p53} were quantified by real-time PCR Taqman assay with \textit{β-actin} as an internal control. These tumor suppressor genes were not frequently deleted in mammary tumors from MMTV-\textit{neu} mice, showing the specificity of the deletion of the \textit{Dmp1} locus. The numbers indicate the ID of each mouse.
Supplementary Figure S5. Expression of p21<sup>Cip1/WAF1</sup> and p16<sup>Ink4a</sup> mRNA in tumors from MMTV-neu mice.

Mammary tumors from Dmp1<sup>+/+</sup>, Dmp1<sup>+-</sup>, and Dmp1<sup>-/-</sup>; MMTV-neu mice were analyzed for the expression of p21<sup>Cip1/WAF1</sup> and p16<sup>Ink4a</sup> mRNA by real-time Taqman assay with β-actin as an internal control. Tumors from wild-type MMTV-neu mice were divided into two groups ND (Dmp1 was not deleted) and HD (Dmp1 showed hemizygous deletion). N: normal mammary gland from a wild-type mouse. The p21<sup>Cip1/WAF1</sup> mRNA was significantly downregulated in tumors from Dmp1<sup>+-</sup> and Dmp1<sup>-/-</sup> mice as well as in wild-type tumors that showed hemizygous deletion for Dmp1. The p16<sup>Ink4a</sup> mRNA was significantly downregulated only in tumors from Dmp1<sup>-/-</sup> mice. Statistical analyses were conducted by unpaired Student’s t tests.
Supplementary Figure S6. HER2 upregulates the hDMP1 mRNA in human breast epithelial cells.

A, real-time PCR Taqman assay of hDMP1 in human breast cancer cell lines. The hDMP1 levels are higher in breast cancer cells with HER2 overexpression. B, specific shRNAs for HER2 downregulate endogenous hDMP1 in human breast cancer cell lines with HER2 amplification. C, HER2 increases the hDMP1 mRNA through the PI3K-Akt-NF-κB pathway. BT-474 and SK-BR-3 cells were treated with specific inhibitors and real-time PCR was conducted for hDMP1. D, effects of inhibition of PI3K (LY294002), Akt (Akt inhibitor IV), or NF-κB (PS341) on the growth and survival of HER2-overexpressing breast cancer cell lines.
CHAPTER III

Prognostic value of the hDMP1-ARF-Hdm2-p53 pathway in breast cancer

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III.1. Abstract

Our recent study showed critical roles of Dmp1 as a sensor of oncogenic Ras, HER2/neu signaling and activation of the Arf-p53 pathway. To elucidate the role of human DMP1 (hDMP1) in breast cancer, one hundred and ten pairs of human breast cancer specimen were studied for the alterations of the hDMP1-ARF-Hdm2-p53 pathway with follow up of clinical outcomes. Loss of heterozygosity (LOH) of the hDMP1 locus was found in 42% of human breast carcinomas, while that of INK4a/ARF and p53 were found in 20% and 34%, respectively. Hdm2 amplification was found in 13% of the same sample, which was found independently of LOH for hDMP1. Conversely, LOH for hDMP1 was found in mutually exclusive fashion with that of INK4a/ARF and p53, and was associated with low Ki67 index and diploid karyotype. Consistently, LOH for hDMP1 was associated with luminal A category and longer relapse-free survival, while that of p53 was associated with non-luminal A and shorter survival. Thus, loss of hDMP1 could define a new disease category associated with prognosis of breast cancer patients. Human breast epithelial cells/cancer cells with wild-type p53 were sensitive to growth inhibition by activated Dmp1:ER while those that delete p14ARF or p53, and/or Hdm2 amplification showed partial or nearly complete resistance, indicating that p53 is a critical target for hDMP1 to exhibit its biological activity.

III.2. Introduction

Breast cancer is the most common malignancy in women and remains significant health issue in industrialized countries (1-3). Strong evidence supports the idea that breast cancer is initiated by defined genomic alterations, many of which are currently
used as therapeutic targets or biomarkers (4). However, it is still unclear which and how
genomic alterations in human breast cancer contribute to its biology. Furthermore, it is
unknown whether they drive progression of the disease, response to therapy, or if they
could be used as prognostic/predictive markers for better patient stratification and
molecular subtyping. Recently, the potential of DNA copy number aberrations for
molecular subtyping of breast cancer has been re-evaluated. It suggests that specific
DNA deletions and/or amplifications may be independent predictors of patient outcomes
apart from analysis of other macromolecules, and warrants future clinical implementation
(5).

Dmp1, a cyclin D binding myb-like protein 1 (also called Dmtf1), was originally
isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as
bait (6, 7). Dmp1 shows its activity as a tumor suppressor by directly binding to
the Arf promoter to activate its gene expression and, thereby, induces Arf- and p53-
dependent cell cycle arrest (8). The activity of the Arf-53 pathway is significantly
attenuated in Dmp1-deficient cells since those cells can easily give rise to immortalized
cell lines that retain wild-type p19Arf and functional p53 and are transformed by
oncogenic Ras alone (9, 10). The murine Dmp1 promoter is efficiently activated by
oncogenic Ras, as well as by constitutively active MEK1/2 and/or ERK1/2 in primary
culture cells (11). Thus, Dmp1 is a key mediator between Ras-Raf-MEK-ERK mitogenic
signaling and the Arf-p53 tumor suppressor pathway.

Dmp1-deficient mice are prone to tumor development. Tumors induced by the
Eμ-Myc or K-Ras transgene were greatly accelerated in both Dmp1+/− and Dmp1−/−
backgrounds with no differences between groups lacking one or two Dmp1 alleles
(9, 10, 12). Indeed, nearly all tumors from Dmp1+/- mice retained and expressed the wild-type Dmp1 allele, and most expressed wild-type Dmp1 mRNA and protein, suggesting typical haploid-insufficiency of Dmp1 in tumor suppression (10, 12, 13-15).

We recently characterized the signaling pathway between HER2/neu and Dmp1 using MMTV-neu mice as a model (16). Both Dmp1 and p53 were induced in premalignant hyperplastic lesions from MMTV-neu mice, and mammary carcinogenesis was significantly accelerated in both Dmp1+/- and Dmp1-/- mice (16). We also observed selective deletion of Dmp1 in >50% of wild-type HER2/neu carcinomas, while the involvement of Arf, Mdm2, or p53 was rare. Tumors from Dmp1-deficient mice showed significant downregulation of Arf and p21Cip1, showing p53 inactivity and more aggressive phenotypes than tumors without Dmp1 deletion (16). Thus, our study shows the pivotal roles of Dmp1 in HER2/neu-p53 signaling and breast cancer development.

The human DMP1 (hDMP1; hDMTF1) gene is located on chromosome 7q21, a region often deleted in human breast/lung cancers and hematopoietic malignancies (17-19). We recently analyzed 51 human non-small cell lung carcinoma (NSCLC) samples and found that loss of heterozygosity (LOH) of hDMP1 was present in ~35% of lung cancers (12) in a mutually exclusive fashion with that of INK4a/ARF and/or p53 in the same samples. This raised the possibility that hemizygous hDMP1 deletion might define a new disease entity with different response to therapy (12, 15). The current study was conducted to demonstrate the frequency and pattern of genes involved in the hDMP1-ARF-Hdm2-TP53 pathway in human breast cancer. We analyzed 110 pairs of normal and cancer tissues from breast cancer for LOH of hDMP1, INK4a/ARF, p53 and gene amplification of Hdm2 (20, 21), and correlated the results of LOH/gene amplification
with disease-free survival and known prognostic markers for human breast cancer (reviewed in 22, 23).

III.3. Materials and Methods

The protocols for LOH assay, statistical analyses, immunohistochemical studies of breast cancer samples, cell invasion assay, and real-time PCR are described in Supplementary Materials and Methods.

*Human breast cancer samples and cell lines*

One hundred and ten pairs of human breast carcinomas and their normal counterparts were obtained from the Tissue Procurement Core Facility of Wake Forest University. The patients’ profiles are as follows. Age: 37–89 years old, mean 57 years; stage I: 30%, stage II: 45%, stage III: 23%, stage IV: 2%; histology, ductal carcinoma (ca): 87%, lobular ca: 6%, metaplastic ca: 3%, mucinous ca: 2%, papillary ca: 2%; HER2, 3+: 22%, 2+: 15%. These cases comprise a population-based cohort treated at Wake Forest Baptist Medical Center from 1999–2008. Standard of care treatments included hormone therapy (i.e., tamoxifen monotherapy), chemotherapy (anthracyclines, taxanes), no systemic therapy, and local radiation. Disease-free survival events were defined as local, regional or distant recurrence during the time interval from diagnosis to last follow-up.

*Classification of human breast cancers*

Breast cancer samples were classified into 5 types (luminal A, luminal B, HER2, triple-negative/basal, and normal/unclassified) based on the data from histochemical
studies for ER, PR, HER2, Ki67, cytokeratin, and morphology of tumor cells as proposed from the Komen Website http://ww5.komen.org/BreastCancer/SubtypesofBreastCancer.html. These pathological examinations have been conducted at Wake Forest University Breast Cancer Center of Excellence. The Ki67 positivity ratio of 14% was used to differentiate luminal A and luminal B subtypes, and breast cancers with HER2 (+++) was categorized into HER2 subtype (27).

*Western blotting*

Proteins were extracted with ice-cold EBC buffer with proteinase inhibitors (7). After gel electrophoresis and transfer to nitrocellulose membranes, proteins were visualized by immunoblotting with affinity-purified polyclonal antibodies to Dmp1 (RAX), p53 (sc-6243G, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Hdm2 (ab16895 [2A10], Abcam, Cambridge, MA, USA), p14ARF (sc-53639, 53640), p21CIP1 (sc-6246), or β-Actin (sc-1615), followed by incubation of the filters with HRP-conjugated second antibodies, and reaction with the enhanced ECL detection kit (PerkinElmer, Boston, MA, USA).

**III.4. Supplementary Materials and Methods**

*Loss of heterozygosity (LOH) and sequencing analyses of human breast cancer specimen*

LOH analyses for hDMP1 (#92465, #198004), INK4a/ARF (#33647, #27251), and p53 (#158111, #89737) were conducted as described previously (Mallakin et al., 2007). Genotypes were identified by peak analysis of the fluorescent signal detected on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The qLOH values
were determined through the following equation. $q_{LOH} = \frac{\text{Area Peak 1}}{\text{Area Peak 2}}$ (normal tissue) divided by $\frac{\text{Area Peak 1'}}{\text{Area Peak 2'}}$ (tumor tissue). LOH was assessed if the $q_{LOH}$ value was found to be $>2.0$ or $<0.5$. Each sample was found to be positive for LOH of the locus when the $q_{LOH}$ values were $>2.0$ or $<0.5$ in one of the two sets of primers. Detailed mapping of the genomic region deleted in breast cancer was conducted using 5 other sets of LOH primers ($\beta$-Actin, D7S644, #69164, #251945, and ABCB1), and by real-time PCR (exons 8 and 20) (Mallakin et al., 2007). For sequencing analyses of cDNAs, total RNA was extracted from breast cancer tissues by using RNALater®-ICE (Ambion/ABI) and TRIzol® (Invitrogen, Carlsbad, CA), and RT-PCR was conducted with PfuUltra™ Hotstart DNA polymerase (Stratagene) to sequence hDMP1 and p53 expressed in breast cancer cells.

As far as previous therapies are concerned, four of 110 breast cancer patients had been treated by chemotherapy in a different hospital. However, this did not change the frequency for alteration of the hDMP1-ARF-Hdm2-p53 pathway. LOH for hDMP1 was found in 42.7% (45/106), INK4a/ARF in 18.9% (20/106), p53 in 34.0% (36/106), and amplification of Hdm2 was found in 12.3% (13/106) in breast cancer without previous chemotherapy. The survival curves in Figure 3 have been created with all the samples except for stage IV.

Statistical Analyses

For each comparison of LOH or amplification of Hdm2, we performed a 1 degree of freedom test to determine whether the LOH of hDMP1, INK4a/ARF, p53, and amplification for Hdm2 in breast cancer samples were more likely to occur mutually exclusively or together. To do this, four separate chi-square tests were performed - one
for each pair of data (i.e., hDMP1 and INK4a/ARF, hDMP1 and p53, hDMP1 and Hdm2, and INK4a/ARF and p53) (Mallakin et al., 2007). In these analyses, we examined the expected cell count versus the observed cell count in order to determine whether there was evidence of mutual exclusivity or not (evidence of mutual exclusivity is supported when the off-diagonal elements of the 2 x 2 table have higher observed counts than expected, whereas mutual exclusivity is not supported if the main diagonal cells of the 2 x 2 table have higher observed counts than expected) (Mallakin et al., 2007). In addition to performing chi-square tests, we estimate 95% confidence intervals for binomial proportions (using a normal approximation) (Mallakin et al., 2007). These intervals were calculated using data where at least one of the two markers showed LOH (or Hdm2 amplification) and we estimated the probability that co-occurrence does not happen given that at least one marker has occurred. The relapse-free survival of breast cancer patients was analyzed by using MedCalc software (Mariakerke, Belgium).

**Human breast cancer cell lines**

Human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). For growth assays, each cell line was infected with either empty vector or Dmp1:ER retrovirus, selected with puromycin, and the resistant cells were seeded at 1x10^5 cells/4 ml. Dmp1:ER was activated with 2μM 4-hydroxytamoxifen (Sigma, St. Louis, MO) (Inoue et al., 1999; Mallakin et al., 2007). The growth curves were created by counting the number of viable cells.

**Immunohistochemical studies of human breast cancer samples**

Immunohistochemical staining of human breast cancers was conducted as described previously (Mallakin et al., 2006, 2007; Sugiyama et al., 2008). These
antibodies were used for immunohistochemistry in formalin-fixed, paraffin-embedded sections: Dmp1 (RAX to amino acids 136 to 150 of Dmp1 [Mallakin et al., 2006, 2010], Ki67 (SP6, Neomarkers), ER (IR657, clone 1D5, Dako [Carpinteria, CA]), PR (IR068, clone PgR636, Dako), and HER2 (sc-284). Antigen retrieval was conducted by standard buffer (S1700) from DAKO. In DMP1 staining, the intensity of staining in normal mammary glands were defined as 1 (+/-; weakly positive) or 0 (-; negative), and then breast cancer tissues were graded into 0 (-), 1 (+/-), 2 (+; moderately positive), and 3 (++; strongly positive). The HER2 quantitation method is the same as that used for all clinical samples of breast cancer and is derived from the "Dako Herceptest scoring system", a well-known and widely accepted method (Jacobs et al., 1999; Tsuda et al., 2002).

**Cell invasion assay**

For MCF7, 50,000 pSR-vector (Oligoengine) or pSR-1131 (shRNA) virus infected cells were plated into upper chamber in serum-free media. In BT-549 cells, 50,000 4-HT-pre-treated (2 μM for 24 hrs) vector:ER or Dmp1:ER virus - infected cells were plated into upper chamber in serum-free media. In bottom chamber complete media was added to act as chemoattractant. Cells were serum starved for 24hrs prior to plating, which were then allowed to invade for 48hrs. Cells that passed through the membrane were stained for quantification. The cells that did not pass through the membrane were scrapped using cotton swab.

**Real-time PCR**

Real-time PCR Taqman assay to quantitate p14ARF mRNA was conducted in an ABI7500 system (Applied Biosystems) using Hs00924091_m1 for ARF and
Hs03023880_g1 for β-Actin internal control. Taqman assays to quantitate genomic DNAs for the hDMP1, INK4a/ARF, p53, and Hdm2 loci were custom-designed at ABI.

III.5. Results

The human DMP1 gene (hDMP1; hDMTF1) is often deleted in human breast cancer

To determine the frequency and patterns of inactivation of the hDMP1-ARF-Hdm2-p53 pathway in human breast cancers, we extracted DNA from 110 pairs of clinical samples and conducted LOH analyses for hDMP1, INK4a/ARF, p53, and gene copy number assay for Hdm2 (exon 4). Representative patterns for LOH-positive cases for each locus are shown in Figure 1. The results from a total of 110 patients are summarized in Table 1 (66 cases with promoter methylation assays for hDMP1) and Supplementary Table S1 (the other 44 cases). LOH for hDMP1 was found in 27 samples with the 5′ probe (#92465, 24.5 %), 30 cases (#198004, 27.3 %) with the 3′ probe, and 46 of 110 cases (41.8 %) with either the 5′ or 3′ probes. None of the 61 samples we studied showed methylation of the hDMP1 promoter (Table 1, the 4th column). None of the 15 randomly chosen breast cancer samples showed mutation(s) for the hDMP1 gene except for the polymorphisms at codon 91 (data not shown). Detailed mapping of the genomic fragment deleted in breast cancer showed that gene deletion was limited to the hDMP1 locus (from #69164 to #251945) (12) in 30 of 32 cases of LOH (93.8%) (Supplementary Figure S1), a higher percentage than hDMP1 deletion in human NSCLC (78.9 %) (12). In one case, the hDMP1 deletion was not detectable by the regular LOH assays since the gene deletion was limited to the exons 8 -20 (case #2005-930) (Table 1).
With INK4a/ARF probes, LOH (including homozygous deletion in #2003-226) was detectable in 19 cases with the 5' probe #33647 (17.3%), 10 cases (9.1%) with the 3' probe #27251, and 22 of 110 (20.0%) with either the 5 or 3' probe. Likewise, LOH for the TP53 locus was detectable in 22 cases (20.0%) with the 5' probe #15811, 30 with the 3' probe #89737 (27.3%), and 37 of 110 (33.6%) with either the 5 or 3' probes. This percentage was higher than the reported percentage of p53 mutations in sporadic breast cancers (20%, 24). We then sequenced the DNA-binding domain of the p53 gene in 10 p53 LOH (+) samples and found that the remaining p53 allele was mutated in 4 of 10 p53 LOH (+) cases (Table 1, Supplementary Figure S2). We then stained tissue blocks from breast cancer (13 p53 LOH [+]) cases and 8 p53 LOH[-] cases) with a specific antibody to p53 (DO-1) and found overexpression of p53 in 6 of 13 p53 LOH(+) cases (46.2%), but not in any of the 8 cases with p53 LOH(-) breast cancers (Supplementary Figure S2). Importantly, all breast cancers with p53 mutation as demonstrated by sequencing showed overexpression of the p53 protein (Supplementary Figure S2). Conversely, none of the 8 samples without LOH for p53 showed high expression of p53 as studied by immunohistochemistry. These results are consistent with the previous report that showed frequent association of p53 mutations with loss of the other p53 allele in breast cancer (25). Thus, the hDMP1 locus was more frequently deleted in our breast cancer samples than the INK4a/ARF or p53 locus.

LOH for hDMP1 and INK4a/ARF was found to be mutually exclusive in 62 of 65 cases (95.4%, \( p = 0.0027, \chi^2 = 8.977; \) 95% confidence interval, 89.8-100%) (Table 1 and Supplementary Table S1). Likewise, LOH for hDMP1 and p53 was also mutually exclusive in 63 of 73 cases (86.3%, \( p = 0.025, \chi^2 = 5.013; \) 95% confidence interval, 78.4-
94.2%). On the other hand, LOH for INK4a/ARF and p53 was exclusive only in 31 of 45 cases (68.9%, \( p = 0.0009, \chi^2 = 11.088 \) against mutually exclusive hypothesis; 95% confidence interval, 55.4-82.4%). The Hdm2 gene amplification (more than 6 copies) was found in 14 of 110 samples (12.7%, Table 1 and Supplementary Table S1). The Hdm2 gene amplification and LOH for hDMP1 appeared to occur independently of the other locus (93.0%, \( p = 0.282, \chi^2 = 1.157 \), not exclusive; 95% confidence interval, 87.1-98.9%). Thus, our data demonstrate that 1) LOH for hDMP1 is typically found in human breast cancers with wild-type INK4a/ARF and p53 genomic loci, 2) LOH for INK4a/ARF and p53 occur simultaneously, and 3) LOH for hDMP1 and Hdm2 amplification occur at random with respect to one another.

We next studied the correlation between LOH for hDMP1 and known prognostic factors for breast cancer: HER2, estrogen receptor (ER), progesterone receptor (PR), Ki67, DNA ploidy, clinical stage, and age (data not shown). Setting the cut off level at 20%, we found significantly more cases with low Ki67 expression (i.e., Ki67+ < or = 20%) in the hDMP1 LOH (+) group in comparison to the LOH (-) group (\( p = 0.0266, \chi^2 = 4.92 \)). Conversely, breast cancers with LOH for p53 were associated with high Ki67 (>20%) (\( p = 0.0153, \chi^2 = 5.88 \)) while LOH for INK4a/ARF or Hdm2 amplification was not associated with this proliferation marker (\( p = 0.196 \) and \( p = 0.522 \) respectively). We also found that breast cancers with LOH for hDMP1 more often had diploid DNA content than LOH(-) cases ( \( p = 0.0463, \chi^2 = 3.97 \)). On the other hand, LOH for INK4a/ARF or p53 was associated with aneuploidy of DNA (\( p= 0.0217, \chi^2 = 5.08; p = 0.0141, \chi^2 = 6.03, \) respectively). Conversely, Hdm2 amplification was not associated with ploidy of tumor DNA (\( p = 0.701 \)). HER2 protein overexpression (2+ -
3+) was found in both hDMP1 LOH (+) (10/41, 24.4%) and (-) (25/59, 42.4%) without a statistically significant difference ($p = 0.064$). This finding is in agreement with the fact that MMTV-neu tumor development was accelerated in both Dmp1-null (16) and p53-mutant (26) backgrounds. There was no statistically significant difference in ER, PR, clinical stage, patients’ age and LOH for hDMP1.

We then classified all the breast cancer cases based on the data from histochemical studies for ER, PR, HER2, Ki67, cytokeratin, and morphology of tumor cells as proposed from the Komen Website http://ww5.komen.org/BreastCancer/SubtypesofBreastCancer.html into luminal A, luminal B, HER2, triple-negative, and unclassified/normal-type (27). The Ki67 positivity ratio of 14% was used to differentiate luminal A and luminal B subtypes, and breast cancers with HER2 (+++) was categorized into HER2 subtype. According to these criteria, 30.8% (32 of 104) of total cases were classified into luminal A, 23.1% (24 cases) were luminal B, 19.2% (20 cases) were HER2 type, 17.3% (18 cases) were triple-negative/basal-type, and 9.6% (10 cases) were unclassified/normal-type (Table 2), which were close to those that had been shown in the literature (27). Six of 110 cases could not be classified due to lack of paraffin sections.

We then conducted statistical analyses and found that hDMP1 LOH (+) breast cancers were significantly associated with luminal A group of breast cancers ($p = 0.0085; \chi^2 = 6.924$) while p53 LOH(+) breast cancers were significantly associated with non-luminal A subtype ($p = 0.0234; \chi^2 = 5.141$) (Table 2). Since LOH for hDMP1 is associated with low Ki67 index, higher incidence of a diploid karyotype, and luminal A subcategory, it was expected that deletion of hDMP1 will be a favorable prognostic factor for breast cancer patients.
Correlation of DMP1 protein expression with hDMP1 LOH and HER2 status in human breast cancer

We then studied whether LOH for hDMP1 affects protein expression in breast cancer samples by immunohistochemistry with specific antibodies (28, 29). The nuclear hDMP1 expression levels were categorized into four grades, 0 to 3++ (Figure 2a). Breast cancer samples without LOH for hDMP1 showed more intense nuclear staining for hDMP1 (mostly grades 2-3) while tumors with LOH showed weaker staining (mostly grades 0-1) ($p = 0.0006$, Figure 2). Normal breast epithelial cells also showed weak (1+) hDMP1 staining (data not shown). We found a significant increase in hDMP1 staining in breast carcinomas that showed HER2 overexpression (2+ or 3+) ($p = 0.0038$, Figure 2b), regardless of LOH for hDMP1. Together, our data show that: 1) hDMP1 protein is downregulated in clinical samples that showed LOH for hDMP1 and 2) HER2 and hDMP1 expression levels are positively correlated.

Impact of LOH for hDMP1, INK4a/ARF, p53, and Hdm2 amplification on breast cancer survival

We then studied the impact of LOH for hDMP1, INK4a/ARF, p53, and Hdm2 amplification in stage I to III patients (n = 108; 2 cases of stage IV patients were eliminated from the survival study, Figure 3). Breast cancers with LOH for DMP1 had longer relapse-free survival than those without LOH ($p = 0.0092$, $\chi^2 = 6.79$; 70% survival 1,987 vs. 1,036 days) (Figure 3a). LOH for INK4a/ARF had no impact on patients’ survival ($p = 0.591$, $\chi^2 = 0.289$; 70% survival 1,121 vs. 1,830 days) (Figure 3b). Conversely, breast cancer with Hdm2 amplification showed significantly shorter survival than those without gene amplification ($p = 0.0217$, $\chi^2 = 5.27$; 70% survival 499 vs. 1,830 days).
days) (Figure 3c). Likewise, LOH for \( p53 \) had significantly negative impact on patients’ disease-free survival \( (p = 0.0211, \chi^2 = 5.41; 70\% \text{ survival 1,036 vs. 1,932 days}) \) (Figure 3d) consistent with the finding that \(~50\%\) of \( p53 \) LOH cases showed simultaneous mutation of the remaining \( p53 \) allele (Supplementary Figure S2). The survival of breast cancer patients without LOH for the three loci and absence of \( Hdm2 \) amplification was not significantly different from those with involvement of the pathway (Supplementary Figure S3). Together, our data indicate that the more downstream the molecule is localized in DMP1-ARF-Hdm2-p53 signaling, the more negative impact the marker shows on breast cancer.

*Growth inhibition of human breast epithelial cells by Dmp1:ER*

Finally, we studied whether conditional activation of Dmp1:ER affects the growth of human breast epithelial and cancer cells. Non-transformed human mammary epithelial cells (MCF10A, human mammary epithelial cells [HMEC]) and breast carcinoma cell lines (MCF7, MDA-MB-175VII, ZR-75-1, BT-549, and HCC1569) were infected with Dmp1:ER or empty vector virus, and puromycin-resistance cells were cultured under the presence of 2 \( \mu \text{M} \) 4-hydroxytamoxifen (4-HT) \( (8, 12) \). The genomic statuses for \( p14^{\text{ARF}} \), \( Hdm2 \), \( p53 \), \( p16^{\text{INK4a}} \), and HER2 for human breast epithelial or cancer cell lines are summarized in Supplementary Table S2. Cell growth was completely inhibited by expressing Dmp1:ER in both MCF10A and \( \text{tert} \)-immortalized HMEC (Figure 4a, b). Significant inhibition of cell growth by Dmp1:ER was also observed in ZR-75-1 (Figure 4e) and MDA-MB-175VII (data not shown) breast cancer cells with wild-type \( ARF \) and \( p53 \) although the effect was significantly weaker in breast cancer cells than in HMEC or MCF10A. Western blotting (and real-time PCR in HMEC) analyses showed significant
accumulation of p14^{ARF}, p53, p21^{CIP1}, and Hdm2 in response to activation of Dmp1:ER in HMEC and ZR-75-1 cells (Figure 5a, e; Supplementary Figure S4). In MCF10A cells, significant accumulation of p53 and p21^{CIP1} was observed at 12-36 hours in response to Dmp1:ER (Figure 5b) although p14^{ARF} did not accumulate due to gene deletion. This data is consistent with our recent findings that Dmp1 physically interacts with p53 to neutralize the activities of Mdm2 in ARF-null cells (30). β-gal staining showed that ~40% of MCF10A cells underwent senescence by Dmp1 while ~70% of HMEC became senescent suggesting mixed growth inhibitory response (Supplementary Figure S5). The growth of MCF7 cells (ARF-null, p53 wild-type) was partially inhibited by Dmp1:ER (Figures 4c). Conversely HCC1569 cells with p53 deletion or BT-549 cells with p53 mutation did not slow down their growth by Dmp1:ER (Figure 4d, f). Indeed lysate analyses showed consistently high levels of p14^{ARF} and undetectable p53 targets p21. We studied the growth of breast epithelial/cancer cells depleted of DMP1 by shRNA (12). Western analyses showed more than 90% downregulation of the hDMP1 protein in all of these three breast cancer cells and inactivation of the p53 pathway in MCF10A (Supplementary Figure S6). Depletion of hDMP1 by shRNA accelerated the growth of MCF7 cells (Supplementary Figure S7), but not T47D or MDA-MB-361 (wild-type ARF, mutant p53), suggesting that endogenous DMP1 is inhibiting the growth of p53 wild-type cells, but not in cells with mutant p53. The growth of p53 mutant cells by shRNA were retarded, possibly because shRNA to hDMP1 affected the function of other splicing variants (31) or hDMP1 interacts with mutant p53 for stabilization.

Then we conducted cell invasion assay using MCF7 cells with or without depletion for hDMP1 (see Supplementary Materials and Methods). Our results show
3.31 +/- 0.603 MCF7 cells with hDMP1 downregulation invaded from upper to lower chamber while only 1.57 +/- 0.970 cells migrated to the lower chamber in mock infected cells ($p = 0.048$). Conversely there was no significant effect of DMP1 expression in invasion assay with p53 mutant BT549 cells (55.2 +/- 9.25 vs. 64.5 +/- 14.1). Together, our data indicate that 1) both non-transformed human mammary epithelial cells and breast cancer cells with wild-type ARF and/or p53 (HMEC, MCF10A, MDA-MB-175VII, and ZR-75-1) are sensitive to growth inhibition/senescence by Dmp1 while breast cancer cells that delete ARF or deleted/mutant p53 show partial (MCF7) or nearly complete (HCC1569, BT-549) resistance to growth inhibitory effect by Dmp1, 2) endogenous hDMP1 inhibits the growth of breast cancer cells with wild-type p53, and 3) DMP1-loss is associated with invasive phenotypes of breast cancer cells.

III.6. Discussion

In this study we analyzed 110 pairs of human breast cancer samples and demonstrated that hDMP1 is deleted in 42% of the cases. This percentage is even higher than the involvement of INK4a/ARF (~20%) or p53 (~35%) of the samples we analyzed, and importantly, was found in mutually exclusive fashion from LOH for INK4a/ARF or p53. On the other hand, LOH for INK4a/ARF and p53 were apparently overlapping, suggesting collaboration of these two loci, possibly through the synergism of $p16^{INK4a}$ loss and p53 inactivation. Deletion of hDMP1 was limited to the hDMP1 locus in 94% cases showing specificity of hDMP1 deletion in breast cancer. Importantly, deletion of the hDMP1 locus resulted in significant downregulation of the nuclear expression of the hDMP1 protein in breast cancer cells, signifying that the gene deletion significantly
affected hDMP1 function and contributed to breast carcinogenesis. DMP1 protein expression was significantly higher in HER2(+) tumors than HER2(-), consistent with our recently published data showing that HER2/neu induces Dmp1 in mouse model of breast cancer and that HER2 activates hDMP1 promoter in human mammary epithelial cells (16).

Our study shows that LOH of hDMP1 is associated with relatively low Ki67 index and increased frequency of diploid DNA, both of which are indicators for favorable prognoses of breast carcinomas (23,32,33). In agreement, hDMP1 LOH(+) breast cancer was associated with luminal A subtype, and relapse-free survival was significantly longer (1,987 vs. 1,036 days) for hDMP1 LOH (+) cases than (-) patients. On the other hand, p53 LOH(+) breast cancer was associated with non-luminal A subtypes, both Hdm2 amplification and LOH for p53 were associated with shorter disease-free survival. Of note, although breast cancers with LOH for hDMP1 was associated with relatively low Ki67 index in comparison to p53 LOH samples, the former samples still showed higher Ki67 index (mean 19% in our samples) than normal breast epithelial cells (~2%; 34), indicating that loss of hDMP1 is associated with proliferation of normal breast epithelial cells, which can collaborate with other genetic alterations to develop breast cancer.

Our study shows that 35% of human breast cancers have LOH for p53 and 46% of such cases have mutation(s) of p53. This means ~16% of breast cancers have mutation(s) for p53 in our samples. Interestingly this percentage of p53 mutation is close to those that have been reported in the literature (20%) in sporadic breast cancers (24). Our data also indicate that approximately half of p53 LOH cases retain one p53 allele without p53 mutation. It has been reported that p53 heterozygous mice develop tumors at a mean
latency of 70 weeks without losing or mutating the wild-type p53 allele in mice (35) suggesting that loss of one allele of p53 contributes to tumorigenesis in vivo. Although we currently do not have enough samples for survival analyses of p53+/− breast cancers, with or without p53 mutation, we continue the study to investigate the impact of single allelic p53 loss with or without p53 mutation on survival of breast cancer patients.

Since hDMP1 is a transactivator for the ARF promoter and p14ARF indirectly regulates the activity of p53 through Hdm2, there is a gradient of prognosis of breast cancer patients from (fair) hDMP1 LOH > INK4a/ARF LOH > Hdm2 amplification > or = p53 LOH (poor) possibly because: i) the closer the molecule is to p53, the more seriously p53 function will be affected, ii) LOH of p53 may be associated with a gain-of-function mutation of p53 (36), and iii) Hdm2 has multiple interacting partners other than p53 (e.g., E2F1, YY1, RB, ribosomal proteins) that explain its oncogenic potential (37). Furthermore, depending on which therapies were used to treat our cohort of patients, it is possible that loss of hDMP1 spared deletion of p53 gene, increased effectiveness of chemotherapy and radiation treatment and, thereby, extended time to relapse.

It should be noted that loss of hDMP1, INK4a/ARF, p53, or Hdm2 amplification did not exclusively correlate with currently used prognostic markers for breast cancer (ER, PR, HER2) (23). Thus, LOH studies for hDMP1, INK4a/ARF, p53, and real-time PCR assay for Hdm2 will be independent laboratory tests to predict the prognosis of breast cancer patients. Although hDMP1-loss is a favorable prognostic factor associated with longer relapse-free survival of patients than hDMP1 intact cases, 35% of breast cancer patients relapsed during the observation period of 8 years. Thus, it is likely that other genetic alteration(s) collaborate with DMP1-loss to accelerate recurrence of the
disease. Further molecular genetic studies are required to clarify which molecular events collaborate with hDMP1-loss in breast cancer progression.

Our data show that shRNA to hDMP1 stimulated proliferation of breast cancer cells with wild-type p53, but inhibited cell growth of cells with mutant p53. There are two possible explanations why p53 mutant cells proliferate slower with hDMP1 knockdown. First, the shRNA used downregulates all the three DMP1 splicing variants including the tumor suppressor DMP1α. The function of other two transcripts is unknown although published study suggested the β and γ variants might be blocking the activity of hDMP1α (31). Development of splicing isoform-specific shRNA will be needed to elucidate the function of each variant on cell growth. The second possibility is that hDMP1 may directly interact with mutant p53 and hDMP1 knockdown may affect p53 gain-of-function, and thereby, reduce proliferative capacity of cells with specific p53 mutation. In support of later, patients with hDMP1 LOH(+) tumors have favorable prognosis compared to patients with hDMP1 LOH(-), half of which harbor p53 mutation, further suggesting that hDMP1 may promote breast cancer progression by stabilizing mutant p53. Thus, it would be of great interest to understand whether DMP1 affects function of mutant p53.

In conclusion, we have characterized the frequency and the pattern of alteration of the hDMP1-ARF-Hdm2-p53 pathway in human breast cancer. Each component in the signaling pathway can define a different disease entity associated with prognosis. Hemizygous deletion of DMP1 is found in nearly half of human breast carcinomas that often retain the wild-type p53 and INK4a/ARF loci. This finding is significant as we move closer towards personalized therapy for each breast cancer patient based on their
tumor genetic alterations. Our data suggests that patients with hDMP1 LOH should be selected for current and future therapies whose efficacy is dependent on an intact p53 gene. On the other hand, patients with wild-type hDMP1 (~50% of all breast cancer patients in this study) in their tumor biopsy should be spared toxic side-effects from treatments that would be ineffective with p53 LOH. Alternatively, further research is necessary to develop small molecules that specifically activate the hDMP1 promoter or protein which will be a feasible approach to treat human breast cancer patients with DMP1 LOH since their tumors maintain a second wild-type DMP1 allele without mutation or promoter methylation.

III.7. Supplementary Discussion

Given previous findings that DMP1 is a tumor suppressor whose loss in mice results in more aggressive tumor phenotypes (Taneja et al., 2010a), it seems paradoxical that DMP1 LOH in human breast tumors is associated with better survival and outcome. It should be noted that one or more components of the hDMP1-ARF-Hdm2-p53 pathway is (are) inactivated in ~80% breast cancers, and those with DMP1 LOH retain wild-type p53, which would be predicted to yield better responsiveness to therapy compared to LOH-negative tumors that have inactive p53. Indeed we have conducted survival analysis using our samples and found that the survival of breast cancers with both hDMP1 and p53 LOH (14%) had the trend to have shorter survival than single hDMP1 LOH cases (70% survival 1,800 days vs. 2,200 days). However, the result was not statistically significant (p = 0.216) due to small number of cases that show LOH for both
hDMP1 and p53. We will analyze more samples to demonstrate the combined effects for hDMP1 and p53 loss on breast cancer survival.

Our results show breast cancers with LOH for p53 and/or INK4a/ARF loci are associated with aneuploidy and high Ki67 index. This is in agreement with a previous report that MMTV- ErbB2 (wild-type); WAP-p53-172H double transgenic mice exhibited dramatically shortened survival than single transgenic ErbB2 mice, with tumors showing anaplastic and aneuploid phenotypes, indicating strong cooperativity of ErbB2 and mutant p53 in tumor development (Li et al., 1997). It was also reported that MMTV-ErbB2; Ink4a/Arf+/− mammary tumors showed increased Ki67 expression, higher expression of cyclin D1, and decreased apoptosis as compared with those from ErbB2; Ink4a/Arf−/− mice (D'Amico et al., 2003). Our recent study shows that mouse MMTV-neu tumors with loss of Dmp1 showed higher histological grades with increased local invasion and higher frequency of metastases and, thus, with more aggressive disease than wild-type tumors without Dmp1 involvement (Taneja et al., 2010a). Thus, inactivation of any component of the Dmp1-Arf-p53 pathway leads to more aggressive phenotypes than those without involvement in mice. The differential impact of DMP1-loss between human breast cancers and mouse mammary tumors can be explained by the fact that most of the breast cancer patients without LOH for hDMP1 showed the involvement of the p53 or the Hdm2 locus (34/64, 53.1%), both of which are associated with poor prognoses of patients (this study; Marchetti et al., 1998; Gasco et al., 2003; Turbin et al., 2006). By contrast, loss/mutation of the p53 gene or overexpression of Mdm2 is rare in mouse mammary tumors from neu-transgenic mice (Taneja et al., 2010a). Since mouse mammary tumors without loss of Dmp1 do not have alterations of these three key
components of the p53 pathway, the phenotypes of Dmp1-deficient tumors are more aggressive than Dmp1 wild-type tumors.

We found that activated Dmp1:ER inhibits the growth of both non-transformed and transformed breast epithelial cells with wild-type p53. However, the biological effects were more prominent in non-transformed breast epithelial cells with wild-type p53 than breast cancer cells with both wild-type ARF and p53 (Figure 4). This is possibly because breast cancer cells have activated other oncogenes that inactivate the p53 (and/or Rb) pathway during the course of cell line establishment. One possible explanation is overexpression of YY1, which is frequently found in breast cancer cells, but not in non-transformed breast epithelial cells such as HMEC or MCF10A (Huang et al., 2011; Zhang et al., 2011). YY1 interacts with all of p53, Hdm2, and ARF proteins and neutralizes the activity of the p53 pathway (Sui et al., 2004), thus it is possible that overexpressed YY1 weakens the effects of Dmp1 even when both p14^{ARF} and p53 are wild-type.

Our study shows an unexpected function of Dmp1 in cell invasion. Published studies showed that deletion of Arf and/or p53 led to actin cytoskeleton reorganization and a significant increase in cell motility (Guo et al., 2003). Reintroduction of the wild-type Arf or p53 genes into Arf-null or p53-null cells reversed the phosphatidyl inositol 3 (PI3) - kinase and Rho GTPase activities as well as the migration phenotype (Guo et al., 2003). The same group also reported that Rho family GTPases cooperated with p53 deletion to promote primary mouse embryonic fibroblast cell invasion (Guo et al., 2004). Thus it is possible that Dmp1-loss promotes cancer cell invasion through regulation of the
PI3K and Rho GTPase activities. Further investigation will be required to demonstrate the roles of Dmp1 in preventing cell invasion.

III.8. Acknowledgments

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References


Supplementary References


Figure 1. Representative patterns of LOH for hDMP1, INK4a/ARF, and p53 in human breast carcinoma.

Genomic DNA was extracted from paired normal and malignant breast cancer specimen and PCR was conducted with 6-FAM-labeled primers that amplify the dinucleotide repeats within (or close to) each locus (Mallakin et al., 2007; Sugiyama et al., 2008). The area peaks of the PCR products were quantitated by ABI 3730xl DNA analyzer. The qLOH values were determined through the following equation: qLOH = Area Peak 1/Area Peak 2 (normal tissue) divided by Area Peak 1'/Area Peak 2' (tumor tissue). The arrows indicate the peak that was lost in tumor cells. The sample was considered to have LOH when the value was >2.0 or <0.5. a, genomic locus of the hDMP1 gene. The two different primer sets were designed to amplify the dinucleotide repeat sequences located on the 5’ and 3’ end of the hDMP1 gene. The non-coding exons were colored silver and the coding exons were colored gold. b, genomic structure of the human INK4a/ARF locus. The two sets of PCR primers were designed to detect the dinucleotide repeats within 500 bps of Exon 1β (#33647) and those between Exon 1β and Exon 1α (#27251). The inverted triangles indicate the location of high-affinity hDMP1-binding sites. c, genomic structure of the human p53 gene and the location of the PCR primers used for LOH analyses. d, LOH analysis of breast cancer with hDMP1 primer sets. 5’: #2006-1202, qLOH = 0.31; 3’: #2004-817, qLOH = 2.05. e, LOH analysis with INK4a/ARF primer sets. 5’: #2008-1476, qLOH = 0.44; 3’: #1999-84, qLOH = 11.25 f, LOH analysis with p53 primer sets. 5’: #2008-1272, qLOH = 0.48; 3’: #2008-26, qLOH = 0.33.
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All samples including Table S1

n=110
95% CI
41.8% 20.0% 95.4% 59.8-100 Exclusive p=0.0027 33.6% 86.3% 68.9% 84.2-84.2 Exclusive Non-excl p=0.025 55.4-82.4 Exclusive p=0.005 12.7% 87.1-98.9 Exclusive p=0.292

96
Table 1. LOH analyses of 66 pairs of human breast carcinomas for the hDMP1, INK4a/ARF, and p53 loci.

Positive results for LOH (qLOH >2.0 or <0.5) are shown in bold red type. When one of the two markers (5' or 3') showed qLOH value >2.0 or <0.5, the sample was considered positive for LOH for the tumor suppressor locus. Cases of mutually exclusive inactivation of hDMP1 and INK4a/ARF or hDMP1 and p53 are shown “yes” in bold blue type. Exclusive of hDMP1: LOH of INK4a/ARF (or p53) or amplification of Hdm2 is not overlapping with LOH of the hDMP1 locus in the same sample. Light brown shading indicates cases with LOH for hDMP1. Detailed analysis by real-time PCR showed that case #2005-930 had an internal deletion for hDMP1 that (darker brown shading). The hDMP1 gene was sequenced in samples with #. The p53 gene was sequenced in samples with * (no mutation), and ** (with mutation). Abbreviations. H.D.: hemizygous deletion as determined by real-time PCR; No del: no deletion by real-time PCR; Un: unmethylated. Homo Del: homozygous deletion; single, LOH was not evaluated due to a single peak result; n.d.: not determined.
Table 2. Subclassification of breast cancers studied and relationship with hDMP1 and p53 LOH.

All the breast cancer cases (n=104, enough information was not available in 6 cases) have been subclassified into luminal A, luminal B, HER2, triple-negative/basal-type, and unclassified/normal-type based on the data from histochemical studies for ER, PR, HER2, Ki67, cytokeratin, and morphology of tumor cells as described in the Materials and Methods. The percentage of our breast cancer samples in each category was very close to those reported in the literature. hDMP1 LOH(+) breast cancers were significantly associated with luminal A category while p53 LOH(+) breast cancers were associated with non-luminal A subtype.
Figure 2. Histological grading of hDMP1 in human breast carcinoma.

a, human breast cancer tissues were stained with Dmp1-specific RAX antibody (Mallakin et al., 2006) and the intensity of the nuclear staining was graded from 3(++), 2(+), 1(+-), and 0 (negative). The scale bar is 100μm. b, correlation between LOH for hDMP1 and immunohistochemical grading of breast cancers. Breast cancer samples without LOH for hDMP1 showed significantly stronger nuclear signals for hDMP1. The hDMP1 signals were significantly higher in HER2 3+ or 2+ samples than in HER2 1+ or negative samples indicating the presence of the signaling pathway between HER2 and hDMP1 in breast cancers. Two different intensity values for hDMP1 indicate that the staining pattern for hDMP1 was mosaic; the average values (DMP1 scores) were used for statistical analyses.
Figure 3. Relapse-free survival of 108 cases of human breast carcinoma dependent on LOH for hDMP1, INK4a/ARF, p53 or Hdm2 amplification.

Kaplan-Meier analyses have been conducted to study the impact of loss or gain of each locus on breast cancer patients’ disease-free survival up to 3,000 days. Only patients with stage I to III disease have been analyzed. Positive cases for gene deletion or amplification are indicated in solid lines and negative cases are shown in discontinuous lines. LOH for hDMP1 (a) has significantly positive impact (i.e. fair prognosis) on patient’s relapse-free survival while Hdm2 amplification (c) or LOH for p53 (d) had significantly negative impact. LOH for INK4a/ARF (b) had little influence on breast cancer patients’ long-term survival.
Figure 4. Proliferation assay of non-transformed human breast epithelial cells and breast carcinoma cell lines that overexpress Dmp1:ER.

(a) HMEC (human mammary epithelial cells); \(HER2^{low}\), \(ARF^+\), \(p53^+\). (b) MCF10A; \(HER2^{low}\), \(ARF^+\), \(p53^+\)

(c) MCF7; \(HER2^{low}\), \(ARF^+\), \(p53^+\)

(d) HCC1569; \(HER2^{amp}\), \(ARF^+\), \(p53^{del}\)

(e) ZR-75-1; \(HER2^{high}\), \(ARF^+\), \(p53^+\)

(f) BT-549; \(HER2^+\), \(ARF^+\), \(p53^{mut}\)

Solid lines show the growth curves of Dmp1:ER virus-infected cells treated with 2 µM 4-HT, discontinuous lines show those of mock-infected cells with 4-HT.

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Figure 5. Western blotting analyses of breast epithelial or cancer cells expressing activated Dmp1:ER or empty vector.

Lysate analyses were conducted by Western blotting with specific antibodies to Dmp1, p14\(^{ARF}\), p53, Hdm2, and p21\(^{CIP1}\). (a) HMEC, (b) MCF10A, (c) MCF7, (d) HCC1569, (e) ZR-75-1, and (f) BT-549 cells. Bottom axis shows hours after addition of 2 \(\mu\)M 4-HT.
Supplementary Table S1. Genomic DNA analyses of 44 pairs of human breast carcinomas (samples not listed in Table 1) for the hDMP1, INK4a/ARF, and p53 loci (LOH) and the Hdm2 locus (gene amplification).

Positive results for LOH (qLOH >2.0 or <0.5) and gene amplification (>3.00) are shown in bold red type. When one of the two markers (5' or 3') showed qLOH value >2.0 or <0.5, the sample was considered positive for LOH for the tumor suppressor locus (Mallakin et al., 2007). ID numbers for hDMP1 LOH plus cases are shaded in light brown. Cases of mutually exclusive inactivation of hDMP1 and INK4a/ARF, hDMP1 and p53, INK4a/ARF and P53, and hDMP1 and Hdm2 are shown “yes” in bold blue type. The abbreviations are explained in the legend for Table 1.
Supplementary Table S2. The genomic statuses of \( p14^{ARF} \), \( Hdm2 \), \( p53 \), \( p16^{INK4a} \), and \( HER2 \) in human breast cancer cell lines used in Figures 4 & 5.

The genomic DNA statuses for these 5 genes are summarized in this table. Het: hemizygous gene deletion; wt: wild-type; mut: mutated; del: homozygous gene deletion; N.D.: not determined.

<table>
<thead>
<tr>
<th>cell line</th>
<th>( p14^{ARF} )</th>
<th>( Hdm2 )</th>
<th>( p53 )</th>
<th>( p16^{INK4a} )</th>
<th>( HER2 )</th>
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<td>wt</td>
<td>wt</td>
<td>wt</td>
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<tr>
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<td>del</td>
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<td>wt</td>
<td>N.D.</td>
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<tr>
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<tr>
<td>ZR-75-1</td>
<td>wt</td>
<td>wt</td>
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<td>low</td>
</tr>
<tr>
<td>MDA-MB-175VI</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>N.D.</td>
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<tr>
<td>BT-549</td>
<td>wt</td>
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<td>N.D.</td>
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</tr>
<tr>
<td>HCC1569</td>
<td>wt</td>
<td>wt</td>
<td>del</td>
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</tr>
<tr>
<td>MDA-MB-361</td>
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<td>amplified</td>
<td>mut</td>
<td>N.D.</td>
<td>overexpressed</td>
</tr>
</tbody>
</table>
Supplementary Figure S1. Detailed mapping of the genomic loci of 7q21 deleted in human breast carcinomas.

The genomic DNA that had been deleted in breast cancer samples were mapped with 7 sets of LOH primers and by real-time PCR for exons 8 and 20 of the hDMPI (hDMTF1) gene. The limits of gene deletion in each patient’s sample are shown in red arrows. The hDMPI (hDMTF1) locus (#69164 - #251945) was selectively deleted in 30 of 32 (93.8%) human breast carcinomas.
Supplementary Figure S2. Mutational analyses of p53 in breast cancer samples.

a. Summary of sequencing analyses of p53 genomic DNA (DNA-binding domain) in breast cancers and immunohistochemical studies with the p53 antibody. The DNA-binding domain of the p53 gene was sequenced in 10 p53 LOH(+) samples. Results show that the remaining p53 allele was mutated in 4 of 10 cases. We also stained tissue blocks from breast cancer in 13 p53 LOH (+) cases and 8 p53 LOH (-) cases with a specific antibody to p53 (DO-1) and found overexpression of p53 in 6 of 13 p53 LOH(+) cases (46.2%), but not in any of the 8 cases of breast cancer without LOH for p53. Importantly, all breast cancers with p53 mutation as demonstrated by sequencing showed overexpression of the p53 protein proving that immunohistochemical staining of breast cancer tissues is a decent method to detect p53 mutation in tumor tissues.

b. Immunohistochemical staining of p53 in breast cancer. The p53 protein was detected by immunohistochemistry using DO-1 monoclonal antibody. Significant amount of p53 was detectable only in cases with p53 mutation as determined by sequencing. Scale bar indicates 100 μM.
Supplementary Figure S3. Relapse-free survival of breast cancer patients that did not show LOH for hDMP1, INK4a/ARF, p53, or amplification of Hdm2.

Kaplan-Meier analyses have been conducted to study the impact of loss (hDMP1, INK4a/ARF, p53) or gain (Hdm2) of each locus on breast cancer patients’ disease-free survival up to 3,000 days. Negative cases for gene deletion of hDMP1, INK4a/ARF, p53, or Hdm2 amplification (n = 22) are indicated in discontinuous brown line and positive cases (n = 86) are shown in solid blue line. There was no significant difference in relapse-free survival between these two groups.
Supplementary Figure S4. mRNA analyses for p14ARF in human breast epithelial cells with activated Dmp1:ER.

Real-time PCR analyses of the ARF mRNA in MCF10A (left) and HMEC (right). Significant induction of p14ARF mRNA by Dmp1:ER was observed in HMEC, but not in MCF10A. The ARF locus was retained in the former, but deleted in the latter.
Supplementary Figure S5. β-Gal staining of mammary epithelial cell lines and breast cancer cells by activated Dmp1:ER.

MCF10A and HMEC cells infected with mock or Dmp1:ER virus were treated with 2μM 4-HT for 48 hrs, and were stained for β-gal to study senescence.
Supplementary Figure S6. Downregulation of the DMP1 protein and Western blot analyses of the p53 pathway in human breast cancer cell lines with shRNA to hDMP1.

Breast cancer or breast epithelial cells were infected with 3 different retrovirus (pSR-Vec: empty vector; pSR-Luc: shRNA to non-mammalian target luciferase; and pSR1131: shRNA to hDMP1 [12]), and puromycin-resistant cells were analyzed for expression of DMP1, hDM2, p53, p21CIP1, and p14ARF. Note that shRNA 1131 depleted more than 90% of the hDMP1 protein in all of these three breast epithelial cell lines. In MCF10A cells with wild-type p53, downregulation of hDMP1 resulted in downregulation of p53, p21CIP1, and hDM2, indicating inactivation of the p53 pathway. Although p53 protein was not detectably expressed in MDA-MB-361 cells, its mRNA was detectable by RT-PCR, indicating the presence of the p53 genomic locus. The hDM2 protein was barely detectable in MDA-MB-361 cells with mutant p53, but was expressed in T47D cells where the hDM2 promoter was mutated and activated by a single nucleotide polymorphism 309. p14ARF was completely eliminated in MDA-MB-361 cells when hDMP1 was knocked down.
Supplementary Figure S7. Downregulation of hDMP1 stimulates cell growth in p53 wildtype breast cancer cells.

Downregulation of hDMP1 mRNA (~80% as determined by real-time PCR) by shRNA accelerated the growth of MCF7 cells with wild-type p53, but not T47D (wildtype ARF, mutant p53) or MDA-MB-361 (wild-type ARF, mutant p53), suggesting that endogenous DMP1 inhibits the growth of p53 wild-type breast cancer cells, but not in those with mutant p53. Possible mechanisms for slower cell growth of hDMP1 shRNA-treated, p53 mutant breast cancer cell lines are explained in the text.
CHAPTER IV

DMP1β, a splice isoform of the tumor suppressor DMP1 locus, induces proliferation and progression of breast cancer

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This chapter was submitted to Cancer Research in November 2013 (Maglic, D., et al. DMP1β, a splice isoform of the tumor suppressor DMP1 locus, induces proliferation and progression of breast cancer. Cancer Research. [submitted]). Stylistic variations within the chapter are due to the journal demands. D.M., K.I., and G.S. designed the study. D.M. executed most of the research with help of P.T., E.A.F, and. M.C. assisted in analysis of the mouse tissues. M.C.W. provided clinical specimens and assisted in pathological analysis. D.M., K.I., and G.S. analyzed the data and wrote the manuscript.
IV.1. Abstract

Our recent work has indicated the *DMP1* locus on 7q21, encoding a haploinsufficient tumor suppressor, is hemizygously deleted at a high frequency in breast cancer. The locus encodes DMP1α protein, an activator of the p53 pathway leading to cell cycle arrest and senescence, and other two functionally undefined isoforms, DMP1β and DMP1γ. In this study, we show that the *DMP1* locus is alternatively spliced in ~30% of breast cancer cases with relatively decreased DMP1α and increased DMP1β expression. Similarly, DMP1β protein was found to be overexpressed in a significant number of tumors relative to their surrounding normal tissue. Importantly, alteration of DMP1 splicing and DMP1β overexpression were associated with poor clinical outcomes of the breast cancer patients indicating that DMP1β may have a biological function. Indeed, DMP1β increased proliferation of non-tumorigenic mammary epithelial cells and knockdown of endogenous DMP1 inhibited breast cancer cell growth. To determine DMP1β’s role *in vivo*, we established *MMTV-DMP1β* transgenic mouse lines. DMP1β overexpression was sufficient to induce mammary gland hyperplasia and multifocal tumor lesions in mice at 7-18 months of age. The formed tumors were adenosquamous carcinomas with evidence of transdifferentiation and keratinized deposits. Overall, we identify alternative splicing as a mechanism utilized by cancer cells to modulate the *DMP1* locus through diminishing DMP1α tumor suppressor expression while simultaneously upregulating tumor promoting DMP1β isoform.

IV.2. Introduction
Breast cancer, as the most common tumor type in women, causes significant health care burden in the Western countries (1, 2). Established biomarkers such as estrogen receptor (ER) and progesterone receptor (PR) play significant roles in the selection and management of patients for endocrine therapies including tamoxifen and aromatase inhibitors. HER2 expression is a strong predictor of response to trastuzumab (3). The roles of ER as a negative and HER2 as a positive indicator for chemotherapy have been established. However, these targeted therapies have not produced the anticipated improvement in long-term patient survival. The initial response is often followed by tumor relapse with intrinsic resistance to the first-line therapy (4-6). In addition, up to 30% of breast cancer patients are overdiagnosed due to implementation of mammography screening with minimal reduction in mortality rates (7). This suggests that many women with early diagnosis of indolent breast tumor may have been spared psychological stress and therapy-associated side-effects if better prognostic/predictive stratification strategies existed. Although proliferation markers such as cyclin D1, cyclin E, p27kip1 have been proposed for molecular stratification of breast cancer, these have not been used as a routine in clinics due to their limited efficacy in deciding therapeutic strategies (3, 8, 9). To develop more reliable biomarkers, it is necessary to further delineate oncogenic events that drive initiation and progression of cancer. An establishment of better prognostic and predictive biomarkers for breast cancer could guide clinical management of patients with a resulting improvement in long-term outcomes and reduced health care cost.

Recently our lab has identified DMP1 (cyclin D-binding Myb-like Protein 1; DMTF1) as a critical tumor suppressor in breast cancer. DMP1 is a transcription factor
that binds directly to the \( p14^{ARF} \) (\( p19^{Arf} \) in mice) promoter and induces its expression. The increase in \( p14^{ARF} \) blocks Hdm2 (Mdm2 in mice)-mediated p53 degradation and leads to cell cycle arrest and senescence (10-12). DMP1 also stabilizes p53 by direct protein-protein interaction to block Hdm2-mediated ubiquitination, which is the major mechanism of p53 activation by DMP1 in \( ARF \)-null cells (13). Loss of heterozygosity (LOH) analysis with specific primers demonstrated that the \( DMP1 \) locus on 7q21 is hemizygoously deleted in \( \sim 42\% \) of breast tumors with mutual exclusiveness to \( INK4A/ARF \) or \( p53 \) loss. The intact \( DMP1 \) allele remained wild-type without promoter hypermethylation (14). Similarly, deletion of \( Dmp1 \) in the \( MMTV-neu \) mouse model accelerated development of the mammary gland tumors without significant difference between \( Dmp1^{+/−} \) and \( Dmp1^{−/−} \) backgrounds suggesting haploinsufficiency of Dmp1 for tumor suppression (11). DMP1 haploinsufficiency was also observed in lymphoma and lung tumor mouse models (15, 16). To date, the molecular mechanisms for Dmp1’s haploid insufficiency remain unknown. Moreover, \( Dmp1^{−/−} \) females are unable to nurse pups due to poor expansion of luminal cells suggesting that the \( Dmp1 \) locus may possess functions other than tumor suppression (11, 17).

The human \( DMP1 \) locus encodes three distinct transcripts via alternative splicing of the \( Exon \ 10 \). The \textit{bona fide} tumor suppressor was named as \( DMP1α \), while two other transcripts with mostly unknown function were named as \( DMP1β \) and \( DMP1γ \). The \( DMP1β \) and \( DMP1γ \) proteins lack the DNA-binding and C-terminal transactivation domains found in \( DMP1α \) and are therefore unable to transactivate \( p14^{ARF} \) or other \( DMP1α \) target genes (Supplementary Fig. S1A and S1B) (18). Unlike \( DMP1γ \) and \( DMP1α \), \( DMP1β \) was found to block differentiation and stimulate monocyte proliferation.
during PMA-induced differentiation to macrophages (18). Hence, the DMP1 isoforms may have unique functions, in particular those other than tumor suppression.

Alternative splicing is a mechanism for a single locus to encode multiple functionally distinct proteins that regulates different biological processes (19, 20). Several splicing factors, RNA-binding proteins regulating alternative splicing, have been identified as proto-oncogenes and are frequently overexpressed in human cancer (21, 22). Multiple cancer-associated genes such as PKM, Bcl-x, CD44, Cyclin D1, p63, and p73 are alternatively spliced in tumors compared to matched normal tissues to produce their tumor promoting isoforms (20, 23, 24). The activities of tumor-associated isoforms vary from regulating novel biological processes to negating the isoforms expressed in normal tissues (25). Since DMP1 is a critical mediator of breast cancer development in humans and mice, we sought to investigate the involvement of the other DMP1 splice isoforms (DMP1β and DMP1γ) in mammary oncogenesis. Using breast cancer cell lines, clinical samples, and a newly established transgenic mouse model of breast cancer, we demonstrate that DMP1 is aberrantly spliced in breast cancer to increase DMP1β and promote disease progression.

IV.3. Materials and Methods

Human breast cancer samples and cell culture

Forty-six pairs of human breast cancer and matched normal tissues in RNALater®-ICE were obtained from the Advanced Tumor Bank of Wake Forest Baptist Hospital with help of Dr. Gregory Kucera. The 48 paraffin embedded breast tumor
blocks were obtained for immunohistochemistry (IHC). The breast cancer samples used in this study (46 for mRNA and 48 for IHC) are within the 110 samples previously described (14). The de-identified patient samples consisted of Stage I (32.6% for RNA; 29.2% for IHC), Stage II (47.8% for RNA; 37.5% for IHC), Stage III (17.4% for RNA; 29.2% for IHC), Stage IV (2.2% for RNA; 2% for IHC), and one patient with unknown stage for IHC. The total RNA from tissues or cells was isolated using TRIzol® Reagent and converted to cDNA using SuperScript® Reverse Transcriptase (Life Technologies) following manufacturer instructions. The LOH analysis of 5’ and 3’ DMP1 loci was performed as described previously (16). MCF10A, BT474, MDA-MB-175VII, and SK-BR-3 cell lines were obtained from the ATCC and cultured in medium according to their recommendation. The MDA-MB-231 cell line was a kind gift from Dr. Linda Metheny-Barlow (Wake Forest School of Medicine). The 3D mammosphere culture was done by plating 4000 cells in diluted Matrigel™ (BD Biosciences) per well in 24-well plates. The cover medium was changed every 3 days and images of mammospheres were taken on day 14 (26). The size of at least 8 mammospheres per condition was measured for the analyses.

RNA analysis and Western blot analysis

TaqMan qRT-PCR analysis was carried out on ABI7500 (Applied Biosystems, Foster City, CA). The TaqMan primers are described in the Supplementary Methods. Mouse Dmp1 copy numbers in MMTV-neu tumors were determined using qPCR as described previously (11). Total proteins from mammary glands or cell lines were isolated using an EBC buffer containing protease inhibitors (Calbiochem proteinase inhibitor cocktail III, leupeptin, AEBSF, and aprotinin) as described before (27). The
following antibodies were used for protein detection: p53 (sc-126), p14\textsuperscript{ARF} (sc-53640), p21\textsuperscript{CIP1} (sc-6246), Hdm2 (sc-812), ACTIN (sc-1615), and BAX (sc-7480) from Santa Cruz Biotechnology, Inc. Pan-DMP1 antibodies (RAD and RAX) were previously described (28, 29). The newly generated DMP1\textbeta antibody is described in Supplementary Methods. Secondary ECL\textsuperscript{TM} Anti-Rabbit/Mouse and Anti-Goat HRP–conjugated antibodies were purchased from GE Healthcare and Santa Cruz Biotechnology, respectively.

*Establishment of MMTV-DMP1\textbeta_{VH} mice*

The V5 and 6\times His tagged human DMP1\textbeta cDNA was cloned into a HindIII site of the *MMTV-SV40-BSSK* vector (a gift from Dr. Philip Leder, Harvard Medical School). After DNA sequencing confirmation, pronuclear microinjection of the targeting vector in the FVB/NJ mouse background was carried out by the Transgenic Core Facility at Wake Forest School of Medicine. The founding offspring were screened and identified by nested PCR, which includes the 1\textsuperscript{st} PCR reaction (Forward: 5’-CTGCCCTCAGAATGAAGCGGATG-3’; Reverse: 5’-GAAACTTCCTCGTTACCCAAG-3’) followed by product purification (Qia gen\textsuperscript{®} Purification Kit) and the 2\textsuperscript{nd} PCR (Forward: 5’-CAGAGTATTGATGATTCTACTCC-3’; Reverse: 5’-GCTCATTCTGCAAAATCTGTATC-3’). The carrier females of the transgene were bred with pure wild-type FVB/NJ males to expand the colonies. The female mice were monitored daily for palpable tumor development. All of the mice were maintained in accordance with an approved IACUC protocol.

*Immunohistochemistry, immunofluorescence, and whole mammary gland mounts*
The mammary glands were fixed in 10% neutral buffered formalin for at least 24 hours. The IHC analysis for human and mouse tissues was carried out as described previously (28). For human IHC, patients with tumors that stained stronger for DMP1β than the surrounding normal tissue were designated as “high” while indistinguishable difference between tumor and normal tissue is indicated as “low.” The following antibodies and their dilutions were used: RAB (1:50), RAD (1:50), Ki67 (SP6, NeoMarkers; 1:200), Cyclin D1 (SP4, NeoMarkers; 1:100), Cytokeratin 8 (ab59400, Abcam; 1:100), Cytokeratin 14 (ab7800, Abcam; 1:50). Whole mammary gland mounts and immunofluorescence analysis using RAB antibody (1:50) was done as described previously (13, 30).

Statistical analyses

Kaplan-Meier graphs for tumor-free survival of MMTV-DMP1β mice and relapse-free survival of breast cancer patients were analyzed by Medcalc software, Mariakerke, Belgium. The following statistical analyses were used in other experiments: two-way ANOVA for the cell growth assays, unpaired Student’s t-test for the mammospheres assays, and two-sided Chi square tests for the DMP1 LOH vs. DMP1β mRNA/protein expression and Supplementary Table 1. A difference was considered statistically significant at $p < 0.05$.

IV.4. Supplementary Materials and Methods

qRT-PCR TaqMan Primer and shRNA Sequences
The human and mouse DMP1α, DMP1β, and DMP1γ isoform specific TaqMan primers were custom designed. DMP1α: Forward 5’-CCATGTGGGAAAAATATACACCTGAAGA-3’; Reverse 5’-CCTATTGTTGCCCAGTCATTGC-3’; TaqMan Probe 5’-CAAGGAGCTCCGGATAAA-3’. DMP1β: Forward 5’-TGATGACAGAAACCATGTGGGAAAAAT; Reverse 5’-CCAAAGTTTGAAAGTGTGGCCTTTT-3’; TaqMan Probe 5’-CAGTTGTTCTCCTTGAGCTTC-3’. DMP1γ: Forward 5’-TGATGACAGAAACCATGTGGGAAAAAT-3’; Reverse 5’-CACAGTTGTCTGTGGGCTTTT-3’; TaqMan Probe 5’-CAGTTGTTCTCCTTGAGCTTC-3’. Mouse Dmp1α: Forward 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-CCAGTCATTGCGCGTGTTTATCC-3’; TaqMan Probe 5’-TCGAGAAGCTCAAGGAGCTCAAGGGAGCT-3’. Mouse Dmp1β: Forward 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-GGTTTGGAAGGTGTGGCCTTTATTGTGT-3’; TaqMan Probe: 5’-CAGTTGTTCTCCTTGAGCTTC-3’. The β-ACTIN assay used as internal control was Hs03023880_g1. The shRNA targeting 3’UTR of DMP1 (DMP1-465) sequences was 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-CCAGTCATTGCGCGTGTTTATCC-3’; TaqMan Probe: 5’-CAGTTGTTCTCCTTGAGCTTC-3’. The shRNA targeting 3’UTR of DMP1 (DMP1-465) sequences was 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-CCAGTCATTGCGCGTGTTTATCC-3’; TaqMan Probe: 5’-CAGTTGTTCTCCTTGAGCTTC-3’. The β-ACTIN assay used as internal control was Hs03023880_g1. The shRNA targeting 3’UTR of DMP1 (DMP1-465) sequences was 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-CCAGTCATTGCGCGTGTTTATCC-3’; TaqMan Probe: 5’-CAGTTGTTCTCCTTGAGCTTC-3’. The β-ACTIN assay used as internal control was Hs03023880_g1. The shRNA targeting 3’UTR of DMP1 (DMP1-465) sequences was 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-CCAGTCATTGCGCGTGTTTATCC-3’; TaqMan Probe: 5’-CAGTTGTTCTCCTTGAGCTTC-3’. The β-ACTIN assay used as internal control was Hs03023880_g1. The shRNA targeting 3’UTR of DMP1 (DMP1-465) sequences was 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-CCAGTCATTGCGCGTGTTTATCC-3’; TaqMan Probe: 5’-CAGTTGTTCTCCTTGAGCTTC-3’. The β-ACTIN assay used as internal control was Hs03023880_g1. The shRNA targeting 3’UTR of DMP1 (DMP1-465) sequences was 5’-GTGGGAAAATACACTCCTGAAGAGA-3’; Reverse 5’-CCAGTCATTGCGCGTGTTTATCC-3’; TaqMan Probe: 5’-CAGTTGTTCTCCTTGAGCTTC-3’.

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**Generation of a DMP1β-specific polyclonal antibody in rabbits**

Polyclonal antibody (named RAB) to the DMP1β/γ was generated by injecting rabbits with KLH-conjugated peptides NH₂-(GC)LWTPKKGHTFKLWLSKCYC-COOH (Bio-synthesis, Lewisville, TX, USA). This sequence corresponds to a.a. 238–256 of the human DMP1β/γ protein. The anti-DMP1β antiserum (RAB) was affinity-purified and was used for Western blotting and immunohistochemical analysis.
Luciferase Assay

NIH3T3 cells were co-transfected with pFLEX1-Flag-Dmp1α, pGL2-Arf, SEAP (chicken β-Actin promoter - Secreted Endocrine Alkaline Phosphatase) and increasing amount of pFLEX1-Flag-DMP1β. Forty-eight hours post transfection, Luciferase activity was measured from cell lysates while SEAP activity was determined from the corresponding media supernatant.

IV.5. Results

DMP1 is aberrantly spliced in breast cancer to overexpress DMP1β

To study whether DMP1 is alternatively spliced in human breast cancer, total RNA from tumors of 20 breast cancer patients and the matched normal tissues were isolated, and qRT-PCR was conducted for DMP1α, DMP1β, and DMP1γ transcripts. The expression of DMP1α was used as internal control to determine DMP1β to DMP1α ($DMP1β/α$) and DMP1γ to DMP1α ($DMP1γ/α$) isoform ratios in each tumor and in its matched normal mammary tissue. To evaluate relative expression of the two DMP1 isoforms among these tissues, we designated the $DMP1β/α$ ratio in the normal tissue #1, which was at the median of 20 normal tissues, as 1.0. The $DMP1β/α$ isoform mRNA ratios were significantly higher (i.e. = or >2.0) in 8 breast cancer samples (~40%) than their matched normal tissues (Fig. 1A), while $DMP1γ/α$ isoform ratios were higher in only 3 (~15%) tumors (Supplementary Fig. S2A). In fact, 11 tumors and their normal tissues had no detectable DMP1γ mRNA, suggesting that DMP1γ unlikely plays a significant role in breast cancer. Hence, we further investigated alteration of DMP1β
expression in breast cancer. The qRT-PCR for an additional 26 patients was conducted to evaluate alteration of \textit{DMP1}β/α isoform expression. When combined with the patients in Fig. 1A, alteration of \textit{DMP1} splicing with increased \textit{DMP1}β/α ratios in the breast tumors versus matched normal tissues was found in 14 out of 46 patients (~30.4%). Importantly, alteration of \textit{DMP1}β/α ratios were found in the patients with wild-type \textit{DMP1} (LOH negative cases) and those with hemizygous deletion (LOH positive cases) \((p = 0.1394, \chi^2 = 2.185)\). This suggests that altered \textit{DMP1} splicing happens in tumors with either two intact \textit{DMP1} alleles or hemizygous deletion. In agreement with human breast cancer, \textit{Dmp1}β/α ratios were high in ~53% mammary tumors from the \textit{MMTV-neu} mouse model regardless of hemizygous deletion of \textit{Dmp1} gene (Supplementary Fig. S2B). Using relapse data, we evaluated the correlation between \textit{DMP1}β/α ratios in tumor and the clinical outcomes of breast cancer patients using Kaplan-Meier survival curves. Patients with high tumor \textit{DMP1}β/α ratios were found to relapse significantly faster than patients with low \textit{DMP1}β/α ratios \((p = 0.047, \chi^2 = 3.952;\text{ Fig. 1B})\). We then studied the correlation between \textit{DMP1}β/α ratios and clinical stages and histological subtypes of breast cancer (Supplementary Table S1). High \textit{DMP1}β/α ratios had the trend to associate with stage I and Luminal A histological subtypes, but neither was statistically significant. In summary, aberrant \textit{DMP1} splicing is present in a significant number of breast cancer cases and carries a biological consequence for the patients.

\textit{The DMP1β protein is elevated in breast tumor tissues and associated with poor patient outcomes}

Next we sought to determine whether DMP1β protein is increased in breast cancer. To detect endogenous DMP1β, we raised a polyclonal antibody against an amino
acid epitope found in the C-terminus of DMP1β and DMP1γ, but not in DMP1α protein (Supplementary Fig. S1A). To determine the specificity of this new DMP1 antibody (named RAB) to different endogenous DMP1 proteins, all three DMP1 isoforms were simultaneously knocked down in MDA-MB-175VII cells using a shRNA (DMP1-465 targeting the 3’-UTR of all 3 isoforms) followed by an immunofluorescent analysis. The fluorescent signal was significantly reduced in the cells expressing the DMP1 shRNA compared to the control cells expressing a shRNA targeting Luciferase (Supplementary Fig. S3A). We further tested the specificity of the RAB antibody against different DMP1 isoforms by individually transfecting each DMP1 isoform into NIH3T3 cells followed by Western blot analysis. While our previously developed pan-DMP1 antibody (RAX) was able to detect all three DMP1 isoforms, the RAB antibody only detected exogenous DMP1β protein (Supplementary Fig. S3B), indicating its high specificity to DMP1β. Using the RAB antibody, we then carried out immunohistochemistry (IHC) with paraffin-embedded tumor tissues from 48 breast cancer patients. The RAB antibody specificity in the IHC analysis was confirmed by the blocked tumor tissue staining after its pre-incubation with 5 µg of the peptide used in the immunization for its production (Supplementary Fig. S3C). The staining intensity of the RAB antibody ranged from low (or undetectable) to high in the tumors compared to surrounding normal breast tissues (Fig. 2A). Specifically, 29 out of 48 (~60%) breast tumors were highly stained by the RAB antibody relatively to the surrounding normal tissues. The staining intensity of the RAB antibody correlated with DMP1β mRNA expression in matched patients (Supplementary Fig. S3D). We graphed a Kaplan-Meier relapse-free survival curve based on high versus low DMP1β staining intensity. The patients with high DMP1β
staining in the tumors relapsed earlier than the patients with low or absent DMP1β ($p = 0.041, \chi^2 = 4.166$; Fig. 2B). There was no correlation between DMP1β protein expression and LOH of the locus, suggesting that these two events are independent ($p = 0.7701$). Breast cancers with high DMP1β protein expression were associated with clinical stage I ($p = 0.0087, \chi^2 = 6.888$), but did not show correlation with any particular histological subtype (Supplementary Table S1). Our data indicate that the DMP1β protein, aside from altered splicing to increase $DMP1\beta/\alpha$ ratios, is frequently overexpressed in breast tumor tissues, and the DMP1β protein expression in breast cancer is associated with tumor initiation and progression. Since breast cancer with high DMP1β expression showed shorter survival, it may be useful as a prognostic indicator to stratify patient outcomes.

_Ectopic expression of DMP1β in breast epithelial cells increases proliferation_

While DMP1α acts as a potent activator of cell cycle arrest and senescence, the $DMP1$ locus can be aberrantly spliced and DMP1β is overexpressed in breast cancer (10, 14, 16). Thus, we set out to determine the biological function of DMP1β in non-tumorigenic cells. The MCF10 cells stably expressing DMP1β or vector alone were used to generate growth curves over 4 days (Fig. 3A, right panel). Whereas our previously published work indicated that DMP1α expression in MCF10A cells inhibited proliferation and induced the p53 pathway (14), DMP1β-expressing MCF10A cells grew significantly faster than the cells with the vector alone (Fig. 3, left panel). MCF10A cells expressing DMP1β formed significantly larger mammospheres than those from the control when plated in a 3D Matrigel™ culture system (Fig. 3B). Similarly, SK-BR-3 (p53 mutant) cells overexpressing DMP1β showed significantly accelerated growth.
indicating that the growth promoting activity of DMP1β may be independent of p53 (Supplementary Fig. S4A). Thus, DMP1β likely has opposing biological roles to DMP1α in non-tumorigenic breast epithelial cells. In summary, while DMP1α activates the p53 pathway and induces senescence (14), the DMP1β isoform increases proliferation of breast epithelial cells in a p53-independent fashion.

**Knockdown of endogenous DMP1 reduces the proliferation of breast cancer cells**

Our previous work has established DMP1α as a tumor suppressor to inhibit breast cancer cell proliferation (14). In contrast, thus far we found that overexpression of DMP1β showed opposing effects on non-tumorigenic cells. To study the consequences of endogenous DMP1β on human breast cancer cell lines, we designed several shRNAs specifically targeting DMP1β and two shRNAs targeting all three DMP1 isoforms. Due to a very limited sequence specific to DMP1β, we encountered difficulty in generating effective shRNAs to specifically silence DMP1β (data not shown). However, the shRNAs (DMP1-1131 and DMP1-465) targeting all three DMP1 isoforms significantly reduced their expression (Fig. 4A-B) (16). The limited sequences unique to each DMP1 isoform prevented us from designing additional isoform-specific shRNAs. The knockdown of all three DMP1 isoforms, independent of mutation or deletion status of the p53 pathway, reduced proliferation of BT474 and MDA-MB-231 (Fig. 4A-B), as well as MDA-MB-175 and ZR-75-1 (Supplementary Fig. S4B, data not shown). Similarly, when MDA-MB-175VII cells with the DMP1 shRNA were plated in 3D Matrigel™ culture, they formed significantly smaller mammospheres than the cells expressing a non-targeting shRNA (Fig. 4C). Knockdown of all three DMP1 isoforms in MDA-MB-175VII cells with the shRNA simultaneously reduced expression of all components of the
p53 pathway (p14<sup>ARF</sup>, p53, p21, Bax) and cell proliferation (Supplementary Fig. S4B). We also observed that DMP1β only partially inhibits DMP1α activity when studied in the Arf promoter activation assay (Supplementary Fig. S5). This finding suggests opposing activity of DMP1 isoforms; while DMP1α functions as an activator of the p53 tumor suppressor pathway to inhibit proliferation, DMP1β increases proliferation of the breast cancer cells.

Establishment of MMTV-DMP1β mouse model

To examine DMP1β function in vivo and whether it has a capacity to induce proliferation of mammary epithelial cells, we set out to establish MMTV-DMP1β transgenic mouse lines. Human V5/6×His-DMP1β cDNA was subcloned into the MMTV-LTR vector and its pronuclear microinjection into fertilized one-cell zygotes (FVB/NJ background) was carried out to create transgenic mice (Fig. 5A). The founder mice were genotyped by PCR and four transgenic females were identified for the colony expansion (Fig. 5A). Western blot and qRT-PCR analyses confirmed expression of transgenic DMP1β protein and mRNA in the mammary glands of MMTV-DMP1β mice (Fig. 5B). In agreement, immunohistochemistry using RAB and pan-DMP1 (RAD) antibodies detected DMP1β protein expression in the luminal cells of transgenic mice (Fig. 5C). Thus, the MMTV-DMP1β mice are viable and express DMP1β protein in the luminal mammary epithelial cells.

DMP1β induces proliferation and mammary gland tumor formation in vivo

Since DMP1β induces cell proliferation in vitro, we predicted that mammary glands from MMTV-DMP1β mice would develop normally with evidence of hyperplasia. Pregnancy in mice significantly alters tumorigenic susceptibility and activity of the
*MMTV-LTR* promoter (31, 32); therefore, we analyzed both nulliparous and multiparous female mice. Parous (n = 19 for transgenics, n = 18 for non-transgenics) and nulli-parous (n = 26 for transgenics, n = 19 for non-transgenics) females have been monitored for mammary lesions/tumor development for 6-20 months. *DMP1β*-transgenic (40% parous) females developed mammary tumors with a mean latency of 16 months (*p* = 0.0001, *χ*² = 19.7818; Fig. 6A-B, upper). Multiparous *DMP1β*-transgenic females developed mammary tumors earlier than non-parous transgenic females (mean latency, 460 versus 545 days, *p* = 0.0052, *χ*² = 7.8233; Fig. 6B, lower). Thus the onset of mammary tumors in *MMTV-DMP1β*-transgenic females was earlier than that of *MMTV-cyclin D1/D3/E, c-rel*, but later than *MMTV-ErbB2* mice (31). We also observed a low incidence (>4%) of pituitary prolactinomas, which have been linked to spontaneous tumors in the FVB strain at an average age of 100 weeks and therefore were eliminated from the analysis (33). As expected, the H&E analysis of the mammary glands from the 18 month old transgenic mice of the four strains showed extensive hyperplasia with multifocal tumor lesions (Supplementary Fig. S6B). The hyperplasia and multifocal lesions were also apparent in the whole mammary gland mounts from the same transgenic female mice (Supplementary Fig. S7). Immunohistochemical studies demonstrated strong staining of the proliferation markers, Ki67 and cyclin D1, in *DMP1β* transgenic mammary glands, which further confirmed the hyperplastic phenotype of the glands (Fig. 6B). The observed tumor lesions were highly infiltrated with immune cells (Fig. 7) and showed evidence of keratinization and squamous differentiation (Supplementary Fig. S6B). To ascertain which cellular compartment of mammary gland proliferated within tumors, we double stained these lesions with antibodies for markers of basal/myoepithelial cells
(Cytokeratin 14; CK14) and luminal (Cytokeratin 8; CK8) cells. The majority of the tumor cells were positive for CK8, suggesting the luminal phenotype of the tumors; however, some tumor cells were positively stained for both CK8 and CK14 (Fig. 7). As expected, the keratinized sheets were exclusively positive for CK14 supporting transdifferentiation phenotype. We observed low cytoplasmic staining for ER and undetectable staining for PR (Fig. 7). The lack of nuclear ER/PR staining indicates that these hormone receptors are not involved in the DMP1β-induced tumor initiation or progression. Hence, an observation that DMP1β has an opposing role with that of DMP1α in vitro was recapitulated in our in vivo mouse model. Overall, while DMP1α functions as a bona fide tumor suppressor to activate the p53 pathway, we provide evidence that the DMP1β splice isoform induces cell proliferation and mammary tumor formation.

IV.6. Discussion

In this study, we have uncovered a novel function of DMP1β, an alternative splicing isoform of DMP1, opposing the activity of DMP1α. The DMP1 locus encodes three unique mRNA transcripts (18), which were found alternatively spliced in breast tumors but not in the matched normal tissues. The splicing alteration increased tumor DMP1β/α isoform ratio in ~30% of breast cancer cases and the DMP1β protein was highly expressed in ~60% of breast cancers; both were associated with poor clinical outcomes of the patients. The other DMP1 isoform, DMP1γ, was rarely increased and often completely absent in the tumor tissues. Hence, our finding supports a notion for the existence of a selective advantage in breast tumors to overexpress DMP1β but down-
regulate DMP1α and perhaps also DMP1γ. Alternative splicing of tumor suppressive genes by cancer tissues has been reported previously. For example, prostate tumors express a fetal pyruvate kinase isoform, PKM2, which promotes aerobic glycolysis, a phenomenon known as the Warburg effect, and stimulates tumor progression (34). DMP1β was previously reported to block PMA-induced differentiation of monocytes to allow continued proliferation, while DMP1γ had little effect in this setting (18). A proof that the Dmp1 locus regulates other mammary gland processes aside from the p53 pathway came from Dmp1-null females as they are unable to nurse offspring due to poor mammary gland development resulting from reduced proliferation of luminal cells (11, 17). In agreement with the role of DMP1β in monocytes, our analysis indicates that patients with high DMP1β/α ratios in their tumors exhibited poor clinical outcomes. By developing a DMP1β-specific antibody, we also show that DMP1β protein is overexpressed in the tumors while maintained at low levels in the surrounding normal tissues.

We have previously reported that DMP1 is hemizygously deleted in the tumors of ~42% breast cancer cases while maintaining the other wild-type allele without promoter hypermethylation (14). The question why loss of one DMP1 allele is sufficient to inactivate DMP1α tumor suppressor activity remained unanswered. Here we provide a possible explanation for this phenomenon by showing increased DMP1β/α ratios occurred in both LOH(+) and LOH(-) cases indicating that tumors may modulate the wild-type DMP1 allele with or without hemizygous DMP1 deletion. Similarly, MMTV-neu tumors had altered Dmp1 splicing in both Dmp1 wild-type tumors and those with naturally occurring hemizygously deleted Dmp1 locus. Thus, the DMP1 locus is
inactivated by two independent mechanisms: 1) hemizygous deletion of the *DMP1α* gene that has tumor-suppressive function (p53-dependent), and 2) altered splicing that increases the DMP1β isoform that has tumor-promoting activity (p53-independent). These two mechanisms can have synergistic effects in tumor development, which needs to be addressed in future experiments by crossing *Dmp1*-knockout mice and *DMP1β*-transgenic mice.

Generation of oncogenic splicing variants from one tumor suppressor locus has been reported for p63 and p73 (23, 24). In both cases, the products of the oncogenic splicing isoforms that lack N-terminal transactivation domains are overexpressed in tumors but not in normal tissues. These ΔN isoforms shows trans-dominant effects on p53 by binding to all p53 family proteins (p53, p63, and p73). In the case of p73, an antibody specific to ΔNp73 was used to demonstrate that tumors overexpressing this isoform correlated with poor clinical outcomes of the patients (35). Interestingly, DMP1, p63, and p73 are all regulators of the p53 pathway. The function of DMP1α is dependent on its ability of stabilizing wild-type p53 via transactivating ARF expression or directly interacting with p53, which inhibits cell proliferation and induces cell cycle arrest in multiple cell types and cancer models (14-17). The DMP1β protein lacks the DNA-binding domain and C-terminal transactivation domain of DMP1α, both of which are necessary for activating the Arf promoter and protein-protein interaction with p53 (13, 36). Therefore, it is unlikely that DMP1β directly modulates the Arf-p53 pathway. Our data suggests that a possible mechanism of DMP1β’s tumor promoting activity is its ability of reducing the activity of DMP1α. If DMP1β’s tumorigenic effect solely acts via inhibiting DMP1α, then *Dmp1*-null and *MMTV-DMP1β* mice should show a similar
phenotype in the mammary gland. However, Dmp1-null female mice rarely develop mammary tumors even after 2 years of age. Thus, DMP1β-regulated oncogenic pathways independent of DMP1α must exist. Full biological activity of the DMP1β is independent of DMP1α-p53 while that of ΔNp63/p73 is dependent on the p53 family members. The detailed mechanism underlying DMP1β’s action deserves further investigation using molecular/genetic approaches and in vivo mouse models.

Our previous work showed that knockdown of endogenous DMP1 using shRNAs targeting all three DMP1 isoforms surprisingly reduced proliferation in two out of three breast cancer cell lines (14). Due to limited isoform specific sequences, we were unable to design effective shRNAs unique to each DMP1 isoform. DMP1 splicing occurs on the Exon 10 where a short sequence containing the TAA stop codon is retained to produce DMP1β and DMP1γ transcripts. The DMP1α-specific shRNAs cannot be designed because the sequence of this transcript is included in all DMP1β and DMP1γ isoforms. Hence, we used shRNAs targeting all three DMP1 isoforms to show reduced proliferation in BT474, MDA-MB-231, MDA-MB-175VII, and ZR-75-1 cells. As expected, knockdown of DMP1α in MDA-MB-175VII (ARF and p53 wild-type) impaired the activity of the p53 pathway. Despite p53 pathway inactivation, these cells still proliferated significantly slower than the control cells, suggesting that DMP1α silencing is not responsible for enhanced cell proliferation. In our rescue experiments, expression of DMP1α with a constitutively active promoter in these and other cell lines is not possible due to activation of the p53 pathway (data not shown) (14). Since overexpression of DMP1β increases cell proliferation independent of p53 status, we
attributed attenuated growth of these DMP1 shRNA-expressing cells to the effect of DMP1β silencing.

A recently developed $MMTV-Dmp1\alpha$ transgenic mouse provided evidence that Dmp1α overexpression was non-tumorigenic but induced the p53 pathway resulting in impaired mammary glands. Additionally, Dmp1α delayed Her2/neu-induced mammary gland tumor initiation, which further demonstrated its tumor suppressor function (37). Conversely, mammary glands from our newly established $MMTV-DMP1\beta$ transgenic mice developed normally without evidence of impairment. Indeed, when analyzed at 7-18 months of age, mammary glands from $MMTV-DMP1\beta$ transgenic mice developed diffuse hyperplasia and multifocal tumors in four independent transgenic strains. The latency for mammary tumor development was much earlier (460 days) in multiparous DMP1β transgenic mice than nulliparous (545 days) females. There was low incidence (>4%) of pituitary prolactinomas, which are associated with spontaneous mammary tumors in the FVB strain around 100 weeks of age (33). The tumors and surrounding glands in the transgenic mice were hyperplastic as they strongly stained by proliferation markers Ki67 and cyclin D1. All of the tumor lesions had evidence of keratinization suggesting that tumors are of adenosquamous phenotype. In fact, the tumor epithelial cells were stained with both CK8 and CK14 suggesting transdifferentiation, while keratin sheets were strongly stained with CK14. Although adenosquamous carcinomas are infrequent in human breast cancer, they are induced in mammary gland by cyclin D1 overexpression and exclusively in $MMTV-cyclin D3$ transgenic mice (38-40). Multiparous $MMTV-cyclin D3$ mice developed adenosquamous carcinomas in mammary glands with average latency of 18 months (39). Likewise tumors from $MMTV-DMP1\beta$
mice, cyclin D3-induced tumors were composed of keratinized sheets with evidence of transdifferentiation. Therefore, it is possible that cyclin D3 and DMP1β converge on the same signaling pathways to induce transformation of mammary epithelial cells.

In this study, we have raised an antibody to the C-terminal region specific to the DMP1β/γ isoform. The RAB antibody recognized DMP1β protein but consistently failed to detect DMP1γ in both Western blotting and immunohistochemistry. Currently, it is unclear why RAB is specific to DMP1β isoform; however, one possibility is that the DMP1γ protein is post-translationally modified at the region that was used for immunization to raise the antibody. Importantly, our data demonstrate that DMP1γ isoform is rarely expressed in human tumor tissues and thus the RAB antibody can be unambiguously used to detect DMP1β isoform in human breast cancer.

In conclusion, we have demonstrated that the DMP1 locus is alternatively spliced to increase DMP1β isoform during mammary oncogenesis, which was associated with breast cancer progression. Our data strongly support that isoform switching at the DMP1 locus observed in these tumors was not a mere reduction in DMP1α expression, but rather simultaneous inactivation of tumor suppressor activity mediated by DMP1α and increase in DMP1β isoform which promotes cell proliferation. Hence, we propose that alternative DMP1 splicing to increase DMP1β/α ratio contributes to haploinsufficiency of DMP1 for tumor suppression. However, the signaling pathway regulating DMP1 splicing in normal and tumor tissues remains unknown. The Exon 10 of DMP1 contains multiple consensus sequences for splicing factors such as SF2/ASF, Tra2-β, and SC35, all of which have been implicated in tumor progression (22, 41-43). It is possible that Her2/neu signaling is involved, as we have observed alteration of Dmp1
splicing in \textit{MMTV-neu}-induced tumors. HER2 activates the PI3K-AKT pathway, which is known to regulate SF2/ASF, an oncogenic splicing factor (44). Moreover, the mechanism of DMP1β-induced proliferation is still unclear. DMP1β lacks necessary domains to function as a transcription factor, and therefore is most likely acting through protein-protein interaction. In fact, DMP1β contains a cyclin D-binding domain whose function is completely unknown. Future studies are needed to dissect upstream signaling that regulates DMP1 splicing in breast cancer and delineate DMP1β interacting partners necessary for its oncogenic activity.

\textbf{IV.7. Acknowledgements}

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References


Supplementary Reference

Figure 1. DMP1 alternative splicing in breast cancer leads to increased DMP1β/α ratios.

A. qRT-PCR analysis for DMP1 splicing in 20 breast tumors versus matched normal breast tissues showing increased DMP1β/α mRNA ratios in 8 patients. Error bars for normal and tumor tissues of each patient represent experimental variations in the real-time PCR analyses. The arrows indicate patients with significantly altered DMP1β/α splicing in tumors compared to their matched normal tissues. DMP1 LOH (loss of heterozygosity) below each patient indicates breast tumors with hemizygous DMP1 deletion. (LOH(+) = one DMP1 allele deletion; LOH(-) = wild-type DMP1 locus). B. Kaplan-Meier relapse-free survival analysis of patients with high DMP1β/α ratios (n=14) versus patients with low DMP1β/α ratios (n=32).
Figure 2. DMP1β immunohistochemistry in human breast tumors.

A. Representative images of DMP1β immunohistochemistry staining from two breast cancer patients. (N = normal tissue; T = tumor tissue). A total of 48 human breast tumors were stained with a DMP1β specific antibody (RAB). DMP1β staining was higher in tumor tissues compared to surrounding normal tissues.

B. A Kaplan-Meier relapse-free survival curve was graphed based on high versus low DMP1β protein intensity. Patients with significantly higher DMP1β (high) staining in tumors compared to the surrounding normal tissue had significantly shorter relapse than the patients with tumors that show undetectable DMP1β (low) in their tumor tissue.
Figure 3. DMP1β expression in non-tumorigenic human breast cell line increases proliferation.

A. Human DMP1β cDNA was stably expressed in MCF10A cells using the pMSCV-puro retroviral vector and growth curves were generated using puromycin resistant cell pools. The DMP1β expression was confirmed by Western blot analysis. Cells expressing DMP1β grew more rapidly (* p < 0.001). B. MCF10A cells stably expressing DMP1β were grown as 3D mammospheres in Matrigel™ for over 14 days when images were taken. Relative sizes of mammospheres between vector and DMP1β expressing MCF10A cells were measured using Image J software (* p < 0.001).
Figure 4. Knockdown of endogenous DMP1 reduces breast cancer cell proliferation.

A-B. BT474 and MDA-MB-231 cells were stably infected with pSUPER.retro.puro retroviral vectors expressing the DMP1-1131 shRNA or a control shRNA. qRT-PCR confirmed the knockdown of endogenous DMP1α and DMP1β isoforms in puromycin resistant cell pools. The cell proliferation was measured by cell counting over indicated time period. C. MDA-MB-175VII cells were infected with retrovirus carrying a shRNA targeting endogenous DMP1 3’UTR (DMP1-465) and puromycin resistant cells were plated in Matrigel™. The growth inhibitory effect of the DMP1 shRNA on mammospheres was quantitated with Image J software and graphed. (* p < 0.001).
**Figure 5. Establishment of MMTV-DMP1β_VH mice and expression of transgene in mammary glands.**

A. A schematic map of the MMTV-LTR targeting construct used for expressing of 6xHis/V5-tagged human DMP1β cDNA. DNA agarose gel analysis of PCR genotyping results shows four transgenic strains (Strains 7, 10, 13, and 22) used for expanding the colonies. B. Western blot analysis shows DMP1β transgene expression in mammary glands. qRT-PCR analysis shows specific expression of the DMP1β mRNA in mammary gland versus other tissues from MMTV-DMP1β_VH transgenic females (MG = mammary gland). C. Immunohistochemical analysis of mammary glands from MMTV-DMP1β and non-transgenic females using DMP1β specific (RAB) and pan-DMP1 (RAD) antibodies.
Figure 6. DMP1β induces mammary gland tumors in vivo.

A. Macroscopic image of palpable mammary gland tumor in MMTV-DMP1β mice. Arrows indicate large tumor mass in the mammary gland. B. Kaplan Meier tumor-free survival curves analyzing DMP1β-transgenic versus non-transgenic and parous versus nulli-parous mice. MMTV-DMP1β mice developed tumors around 16 months of age (upper). The tumorigenic incidence in DMP1β-transgenic mice was accelerated when females went through several rounds of pregnancy (lower). C. Representative histological analysis of mammary glands from MMTV-DMP1β and non-transgenic mice at 18 months of age. The transgenic mammary glands show evidence of hyperplasia with focal tumors, immune cell infiltrate, and keratinized deposits indicative of adenosquamous carcinoma. Black arrows indicate areas of keratinization, while red arrows indicate inflammatory cell infiltrate. Proliferative markers, Ki67 and cyclin D1, were overexpressed in mammary glands from transgenic females.
Figure 7. Characterization of mammary tumors from *MMTV-DMP1β* mice.

Immunohistochemical staining of tumor lesions from two transgenic strains for Estrogen receptor (ER), Progesterone receptor (PR), and double staining for Cytokeratin 8 (CK8; brown) and Cytokeratin 14 (CK14; blue). CK8/CK14 double staining indicates that tumor epithelial cells mostly express luminal marker (CK8) while some cells express the basal/myoepithelial marker (CK14). Double CK8/CK14 staining is indicative of transdifferentiation. Black arrows indicate keratinized sheets strongly stained with basal cell marker (CK14). Red arrows indicate immune infiltrate surrounding tumor cells. The ER staining was weak and mostly cytoplasmic, indicating non-active receptor. The PR staining was completely absent in tumor cells.
Supplementary Figure S1. Domain structure of DMP1 splice isoforms.

A. Protein domain structures of DMP1 splice isoforms indicating lack of DNA binding domain and C-terminal transactivation domain in DMP1β and DMP1γ isoforms. The green box shows unique amino acid sequence found in DMP1β and DMP1γ. The DMP1 isoform specific antibody (RAB) was developed using an epitope sequence (NH3-LWTPKKGHTFKLWLSKYC-COOH) in the green box. B. Sequence of DMP1 Exon 10 showing the splice donor and acceptor sites that generate DMP1α, DMP1β, and DMP1γ proteins. DMP1β and DMP1γ unique portions of Exon 10 contain the TAA stop codon.
Supplementary Figure S2. Alteration of DMP1 splicing in human breast cancer and mouse mammary tumors.

A. qRT-PCR analyses for alteration of DMP1γ/α splicing in 20 human breast tumors versus matched normal breast tissue showing infrequent increase in DMP1γ mRNA. The error bars for normal and tumor tissues of each patient represent experimental variations in the real-time PCR analyses. The arrows indicate patients with aberrant DMP1γ/α splicing in tumors compared to their matched normal tissues. DMP1 LOH (loss of heterozygosity) below each patient indicates breast tumors with one copy deletion of DMP1. B. qRT-PCR of mammary tumors from MMTV-neu mice to detect alteration of Dmp1β/α ratios in tumor tissue compared to the normal mammary glands. Tumor were separated into those that retain wild-type Dmp1 (ND) and those that have naturally deleted one allele of Dmp1 (HD). NMMG = normal mammary glands; ND = non-deleted Dmp1 tumors; HD = hemizygous Dmp1 deleted tumors. The red bars indicate mouse tumors with aberrant Dmp1β/α ratios when compared to the normal mammary glands. The error bars indicate for normal and tumor tissues represent experimental variations in the real-time PCR analyses.
Supplementary Figure S3. Evaluation of a newly generated DMP1 splice isoform-specific antibody (RAB).

A. Immunofluorescence analysis of endogenous DMP1β in MDA-MB-175VII cells expressing DMP1 shRNA. The DMP1β specific antibody (RAB) shows reduced detection in DMP1-465 shRNA-expressing cells compared to those expressing a control shRNA targeting Luciferase. The signal intensity for DMP1β (green) was quantified to be 3.25ms exposure for control cells compared to 11.1ms for DMP1-465 shRNA-expressing cells. 

B. NIH3T3 cells were transfected with vector, DMP1α, DMP1β, or DMP1γ cDNA constructs. Western blot analysis shows that the pan-DMP1 antibody (RAX) detects all three isoforms, including the endogenous Dmp1 in the vector transfected cells, while RAB detects only DMP1β protein (* corresponds to a non-specific band). 

C. Representative RAB immunohistochemistry from two patients showing strong staining that was abolished when the RAB antibody was pre-incubated with 5µg of the peptide used for immunization of raising the antibody. 

D. qRT-PCR analysis of DMP1β mRNA expression in breast tumors compared to matched normal tissues indicates positive correlation between DMP1β mRNA and RAB antibody IHC staining.
Supplementary Figure S4. Knockdown or overexpression of DMP1β modulates proliferation of cells independent of the p53 pathway.

A. Overexpression of DMP1β using pMSCV-puro retroviral vector in a p53 mutant cell line, SK-BR-3, increases cells proliferation. Western blot analysis confirms expression of DMP1β protein in the puromycin resistant cells. B. Western blot analysis for DMP1α, hDM2, p53, p21, p14ARF, and BAX shows inactivation of the p53 pathway in MDA-MB-175VII cells expressing DMP1 shRNA (pSR-DMP1-465; targeting all three DMP1 isoforms) compared to the cells expressing non-targeting control shRNAs (pSR-Vec, pSR-Luc, pSR-168). The p53 pathway inactivity is a result of DMP1α knockdown. Growth curve of MDA-MB-175VII cells expressing DMP1-465 shRNA shows reduced proliferation despite inactivation of the p53 pathway. The qRT-PCR analysis confirms reduction of DMP1α and DMP1β mRNA in cells expressing pSR-DMP1-465 shRNA (*p < 0.01).
Supplementary Figure S5. DMP1β partially inhibits DMP1α activity on the Arf promoter.

Co-expression of DMP1β in NIH3T3 cells reduces DMP1α-mediated activation of the Arf promoter in a dose dependent manner. The DMP1β also reduces activity of the Arf promoter in the absence of ectopic DMP1α expression. The expression of DMP1α (100ng per 60mm dish) was kept constant while DMP1β expression was increased (100ng, 200ng, and 300ng of plasmid per 60mm dish). SEAP co-transfection in each plate served as the control for transfection efficiency (* p < 0.05).
Supplementary Figure S6. Mammary gland phenotype of 18 month old MMTV-DMP1β female mice.

H&E stain of mammary glands from 4 different MMTV-DMP1β mouse strains showing adenosquamous carcinoma phenotype. The arrows indicate areas of keratin sheet deposition.
Supplementary Figure S7. Whole mammary gland mounts from MMTV-DMP1β female mice.

Representative whole mammary gland mounts from two 18-month old MMTV-DMP1β (upper 2 images) female mice and a non-transgenic (lower image) female mouse. Mammary glands from MMTV-DMP1β mice show diffuse hyperplasia and multifocal lesions compared to non-transgenic mammary glands. White arrows indicate tumor lesions.
Supplementary Table S1. Correlation of DMP1β expression with current histological and molecular classifications of human breast cancer.

Currently used prognostic indicators, clinical stage and sub-classification, for breast cancer were correlated to DMP1β protein (IHC) expression and aberrant DMP1β/α splicing. High DMP1β protein staining in IHC was found associated with stage I of breast cancer ($p = 0.0087$, $\chi^2 = 6.888$). Aberrant DMP1β/α (high) ratios were found to trend with stage I but the data were not statistically significant.
MEKK1 regulates the Dmp1-Arf-p53 pathway and is frequently inactivated in breast cancer

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This chapter has not been published. D.M., G.S., and K.I. designed the study. D.M. carried out most of the experiments. R.D.K. and D.P.F. contributed in discussions and helped in various experimental techniques. L.D.M. analyzed breast cancer microarray data. D.M. composed the chapter with editorial assistance of G.S.
V.1. Abstract

Dmp1 (cyclin D-binding myb-like protein 1) is a potent transcription regulator of Arf and an activator of the p53-dependent cell cycle arrest. Recently, our lab has shown that Dmp1 promoter is activated by oncogenic HER2/neu. In fact, DMP1 is hemizygoously deleted in ~42% of human breast cancer cases, mutually exclusive with deletion of the INK4a/ARF or p53. Hence, Dmp1 is a critical tumor suppressor in breast cancer that prevents early oncogenic transformation. Aside from promoter regulation, Dmp1 protein is post-translationally modified including via phosphorylation. Currently, it is unknown how phosphorylation of Dmp1 affects its function in normal or cancer biology. Here, we identify a Serine/Threonine kinase of the MAPK pathway, MEKK1, which increased Dmp1 transcriptional activity. MEKK1 expression in normal cells was sufficient to induce the p53 pathway and senescence. In a series of attempts to map the MEKK1 phosphorylation sites on the Dmp1 protein, we discovered that the MEKK1-mediated increase in Dmp1 transcriptional activity is dependent on the MEKK1 kinase activity but independent of direct Dmp1 phosphorylation. MEKK1 increased accumulation of the Dmp1 protein without an effect on the Dmp1 mRNA. In human breast tissues, the MAP3K1 locus on 5q11 was hemizygoously deleted in ~20% of patient’s tumors analyzed. Importantly, low MEKK1 expression correlated with increased distant metastasis. Overall, we identify a Serine/Threonine kinase, MEKK1, which increased Dmp1 transcriptional activity via protein accumulation. The MEKK1 encoding locus, MAP3K1, was deleted in a significant number of breast tumors and its expression predicted patients’ clinical outcome.
V.2. Introduction

Breast cancer is the most common malignancy in women (1). Although a lot of research has been done to understand the biology of this disease, unsubstantial improvements have been implemented in the clinic (2). Furthermore, physicians still rely on the cytotoxic and anti-estrogen agents that have been in use for the past several decades for the treatment of breast cancer (3-4). Very few novel and useful therapeutic agents have been developed in the recent years. The individuals with a hormone-driven disease are deemed to have good prognosis and are most commonly treated with a form of anti-estrogen adjuvant therapy (5). On the other hand, patients with estrogen-negative disease, amplification of \( \text{HER2} \) or \( c-Myc \) oncogenes, and mutation of \( p53 \) gene have poor prognosis and are treated with cytotoxic and anti-receptor antibody adjuvant therapy (6). Importantly, within both groups of the poor and good prognosis, there exists a patient population that achieves full response, while others undergo relapse (7-8). It has been suggested that differential response among breast cancer patients is due to enormous disease heterogeneity with complex genomic alterations (9-12).

Recently, our group began to investigate role of tumor suppressor Dmp1 in breast cancer. Dmp1 (cyclin \( D \)-interacting myb-like protein 1) is a transcription factor isolated in yeast two-hybrid screen as a cyclin D2 binding partner (13). As a transcription factor, Dmp1 binds to nonameric DNA consensus sequences \( \text{CCCG(G/T)ATG(T/C)} \) to act as an activator or a repressor (14). Extensive molecular and genetic evidence links tumor suppressor function of Dmp1 to regulation of the ARF-p53 pathway. In fact, Dmp1 directly binds to the \( p19^{\text{Arf}} \) (\( p14^{\text{ARF}} \) in humans) promoter and induces its expression. Increase in ARF level leads to protection of p53 from Mdm2 mediated degradation and
subsequent increase in p53 target genes involved in cell cycle arrest and apoptosis (15-16). Dmp1-null mouse embryonic fibroblasts (MEFs) do not undergo replicative senescence after many passages and retain intact p19\(^{\text{Arf}}\) and p53. Moreover, Dmp1-null MEFs can be transformed with the mutant Ras alone while maintaining low levels of p19\(^{\text{Arf}}\) and p53 (17). Loss of Dmp1 predisposes mice to development of wide varieties of tumors including lung, mammary, and lymphomas (17-18). In vivo studies on Dmp1’s role in tumorigenesis have shown that oncogenic Ras activates Dmp1 promoter through MAPK pathway (19). Importantly, expression of mutant Ras in the Dmp1-null background significantly accelerated development of lung tumors, without difference between Dmp1\(^{-/-}\) and Dmp1\(^{+/-}\) cohorts. This suggests that Dmp1 is a haploinsufficient tumor suppressor in the Ras model of lung tumorigenesis (20). More recently, we have characterized the role of Dmp1 in development of breast tumors. Similarly to Ras model of lung cancer, Dmp1 is efficiently activated by HER2/neu oncogene in the mammary tissue; however, it is dependent on the PI3K-Akt-NF-κB pathway. Development of mammary tumors in response to HER2/neu oncogene was shortened significantly in the Dmp1-null background with the evidence of haploinsufficiency (21).

Human DMP1 gene is located on 7q21 chromosome, a locus frequently deleted in many different cancers (22-24). Studies from our laboratory using human breast and lung cancer samples have shown that DMP1 locus is disrupted in ~42% and ~35% cases, respectively (20, 25). Specific loss of DMP1 was mutually exclusive with deletion of INK4A/ARF and/or p53. This suggests that DMP1 is a physiological regulator of the ARF-p53 pathway in both humans and mice. DMP1 deletion associated with the Luminal A subtype of breast cancer, lower Ki67 index, and better overall patient outcome
when compared to the patients that retained both \textit{DMP1} alleles (25). Therefore, our published data provide strong evidence for DMP1 as a critical mediator of the ARF-p53 pathway in breast cancer.

Based on the amino acid sequence, Dmp1 protein is expected to migrate to \(~85\text{kDa}\) in a SDS-PAGE gel; however, it is most often observed between 120 and 130\text{kDa} (13). This suggests that the Dmp1 protein undergoes extensive post-translational modification whose role has not been previously investigated. When Dmp1 was initially isolated, it was observed that its binding partner, cyclin D, and Cdk4 directly phosphorylate the Dmp1 protein (13, 15). The function of this modification was never further investigated. Using a 2D SDS-PAGE gel and treatment with the calf-intestinal phosphatase (CIP), we have also shown that one mode of Dmp1 post-translational modification is via phosphorylation. The presence of phosphates on several Serines and/or Threonines was confirmed with mass spectrometry. Furthermore, we identified the MEKK1 kinase that increased Dmp1 activity on the \textit{Arf} promoter, which was abolished with expression of the kinase deficient MEKK1 mutant.

MEKK1 is a Serine/Threonine kinase whose function has been best characterized in the context of inflammatory signaling (26). MEKK1 is activated in response to TNF-\(\alpha\) and IL-1 through their distinct receptors (27). Certain cytotoxic drugs, such as cisplatin, mitomycin C, and etoposide, also activate MEKK1 (28-30). Activation of MEKK1 is dependent on its autophosphorylation and subsequent cleavage of the N-terminal regulatory domain to release active C-terminal catalytic domain (31-32). Active MEKK1 can directly phosphorylate many different downstream effectors including MEK1, M KK4/7, and IKK (33-35). Current evidence suggests that full length 195\text{kDa} MEKK1,
localized at the membrane, can be cleaved at Asp874 by activated caspase-3 to produce the catalytically active 91kDa C-terminal fragment (32, 36). The C-terminal and catalytically active fragment of MEKK1 (from here on referred to as the CA-MEKK1) can potentiate further cleavage of caspase-3 and induce apoptosis (37). Furthermore, catalytically active fragment can translocate into nucleus to participate in the release of co-repressors from androgen and estrogen responsive elements and thereby increase expression of the hormone regulated genes (38). Importantly, MEKK1 (MAP3K1) is located on the human 5q11 chromosome, a locus frequently deleted in many different tumor types including breast cancer (39-40). Role of MEKK1 in the context of cancer has not been studied and little is known how this molecule participates in tumorigenesis. More recently, series of high-throughput sequencing studies discovered MAP3K1 mutations in 8% of breast cancers, which associated with the Luminal A subtype. The MEKK1 mutations were suggested to be inactivating since they clustered in the kinase domain (9-11). Since MEKK1 increased activity of the tumor suppressor Dmp1 in vitro and both genes were frequently inactivated in the Luminal A subtype of breast cancer, we instigated MEKK1 cooperation with Dmp1 during tumor suppression.

V.3. Materials and Methods

Cell Culture

NIH3T3 and mouse embryonic cells were cultured as described previously (16). Human IMR-90 fibroblasts were obtained from ATCC and cultured according to their recommendations. For the preparation of retroviruses or lentiviruses, 293T cells were co-
transfected with pMSCV-IRES-puro or ΔCCR-puro vectors and the helper retroviral or lentiviral plasmids (16, 41).

**Western blot, immunoprecipitation, and qRT-PCR**

Cells were lysed in the ice-cold EBC buffer containing the protease/phosphatase inhibitors followed by sonication and lysate clearing with 20,000g centrifugation for 10 minutes at 4°C (19). Total protein concentration was measured via BCA assay and sample buffer was added followed by boiling for 5 minutes. Dmp1 and MEKK1 proteins were immunoprecipitated using 250μg of protein lysate per IP and 2μg of the corresponding antibodies by sample rotating for 2 hours at 4°C. Protein G-Sepharose (Life Technologies) was used to pull down the antibody-protein complex followed by 4 washes with ice-cold EBC buffer. The sample buffer and boiling for 5 minutes was done to dissociate the antibody-protein complex. The following antibodies were used for Dmp1 detection: RAX, RAD, and Flag M2 (Sigma) (42-43). MEKK1 antibodies used were: sc-252 and sc-473 from SantaCruz Inc. Other antibodies used were: p21^{CIP1} (sc-6246), p53 (sc-6243), Actin (sc-1615) from Santa Cruz Biotechnology Inc. and p19^{Arf} (ab80) from Abcam. Total RNA from cells was isolated using TRIzol® and converted to cDNA using SuperScript® III reverse-transcriptase following the manufacturers protocol (Life Technologies). The inventoried TaqMan primers were purchased from ABI. qRT-PCR analysis was carried out on ABI7500 (Applied Biosystems, Foster City, CA).

**Luciferase Assay and In Vitro Kinase Assay**

Reporter assays were conducted using a previously described murine Arf promoter upstream of cDNA encoding a Luciferase in the pGL2 vector (15). Chicken β-
Actin promoter driven Secreted Endocrine Alkaline Phosphatase (SEAP) was co-transfected with all construct to serve as a transfection efficiency control. The measurement of luciferase and SEAP activities was done as described previously (15). The recombinant CA-MEKK1 was purchased from Sigma while the murine Dmp1 was purified from bacteria (43). The *in vitro* kinase assay was carried by incubating 0.5μg of recombinant CA-MEKK1 with Dmp1 in 30μl of kinase buffer at 30°C for 30 minutes. The kinase buffer contains 20mM HEPES pH 7.5, 20mM β-glycerolphosphate, 10mM MgCl₂, 100mM Na₃VO₄, 20μM rATP, and 5mCi of [γ³²P]rATP. The reaction was stopped by addition of sample buffer and boiling for 5 minutes. The phosphorylation was detected by running samples on a SDS-PAGE followed by drying of the gel and directly exposing the gel to a radiography film.

*Confocal Microscopy*

NIH3T3 cells expressing HA-tagged wild-type or phospho-deficient Dmp1 mutants were plated in Lab-Tek 4 well chamber slides. Detailed method for the immunofluorescent analysis was described previously (43). The wild-type and phospho-deficient Dmp1 mutants were detected using anti-HA antibody (sc-805, Santa Cruz Biotechnology Inc.) at 1:50 dilution. Nuclear DNA was detected with To-Pro-3 diluted at 1:1000. After cover slips were mounted using the ProLong Gold anti-Fade reagent (Invitrogen) on the slides, slides were allowed to cure for 24 hours in a dark and cold environment. Dmp1 localization was analyzed using a laser scanning confocal microscope (Zeiss LSM510).

*LOH analysis of human breast cancers*
The qLOH analysis and breast cancer patient population used in this study was previously described in detail (25). The primer sequence used to amplify 3’ end of the MAP3K1 gene was Forward: 5’-[6FAM]GCCCTTTCTCCTGGCTTCTTGGT-3’, Reverse: 5’-TGGACATTGAGGTAGTCAGCA-3’.

**Mass spectrometry analyses**

Flag-tagged Dmp1 was transiently expressed alone or with CA-MEKK1 in NIH3T3 cells and immunoprecipitated with anti-Flag M2 beads (Sigma) as described above. The bound Flag-Dmp1 protein was eluted from the beads using 20 fold excess of Flag M2 peptide (Sigma). The mass spectrometry analysis was outsourced and performed by the laboratory of Dr. John M. Koomen at Moffit Cancer Center, Tampa, Florida.

**Statistical analyses**

Statistical analyses for human breast cancer patients utilized the Kaplan-Meier survival analysis and the two-sided Chi square tests for mutual exclusive analysis. Other statistical differences were calculated using unpaired Student’s t-test. A difference was considered statistically significant at \( p < 0.05 \).

**V.4. Results**

**Dmp1 protein is modified via phosphorylation on multiple Serine and Threonine residues.**

Current evidence suggests that Dmp1 protein is post-translationally modified. The type of post-translational modification or its effect on the Dmp1 function is
Phosphorylation algorithms predicted several Serine (S) and Threonine (T) residues in the Dmp1 protein to have a potential for phosphorylation. Specifically, we observe a high confidence level for phosphorylation of the Serine or Threonine residues followed by a Proline (SP: Serine-Proline or TP: Threonine-Proline motifs), the consensus motifs for canonical MAPK signaling pathway phosphorylation. Hence, we first investigated whether phosphorylation of Dmp1 protein can be detected in NIH3T3 cells. To detect phosphorylation, Flag-tagged Dmp1 was transiently expressed and immunoprecipitated followed by treatment with the calf-intestinal phosphatase (CIP) to nonspecifically remove all phosphates on the protein. After running a 1D western blot, we observed increased mobility of the CIP-treated Dmp1 protein compared to a mock treatment, which indicated presence of phosphorylated residues. The shift in Dmp1 protein following de-phosphorylation was even more apparent in a 2D Western blot (Supplementary Fig S1A). This data indirectly suggests modification of the Dmp1 protein via phosphorylation. To gain more direct evidence of baseline Dmp1 phosphorylation and which residues are modified, Dmp1 was immunoprecipitated from NIH3T3 cells, and subjected to the liquid-chromatography and mass spectrometry (LC-MS). The mass spectrometry analysis identified presence of phosphorylation on multiple Serine and Threonine residues (Supplementary Fig. S1B; right panel; red lettering). Importantly, most of the sites identified in the mass spectrometry analysis coincided with the SP or TP consensus sequences phosphorylated by the MAPK signaling pathway (Supplementary Fig S1B, left panel) (44). Thus, Dmp1 protein is most frequently phosphorylated on the S or T residues corresponding to the MAPK consensus phosphorylation sites.
A Serine/Threonine kinase of MAPK pathway, MEKK1, increases Dmp1 transcriptional activity.

Dmp1 protein is phosphorylated on multiple S and T residues, but the role of this modification remains unclear. In an unrelated experiment, we observed increased Dmp1 transcriptional activity on the p19\textsuperscript{Arf} (Arf) promoter when co-expressed with the constitutively active MEKK1 (CA-MEKK1). MEKK1 is a Serine/Threonine kinase of the MAPK signaling pathway that phosphorylates its substrates on SP or TP motif, a consensus sequence most commonly phosphorylated on the Dmp1 protein (45). Indeed, we confirmed dependence of MEKK1 kinase activity in the Dmp1-mediated activation of the Arf promoter. Expression of the kinase-dead MEKK1 mutants (K1253M or D1369A) failed to increase Dmp1 transcriptional activity observed with the CA-MEKK1 (Figure 1) (46-47). To study MEKK1-Dmp1 cooperation on the activation of endogenous Arf and p53 proteins, we infected wild-type mouse embryonic fibroblasts (MEFs) with a retrovirus carrying the CA-MEKK1 cDNA and selected stable clones for puromycin resistance. The cells expressing CA-MEKK1 arrested growth and acquired morphological changes indicative of senescence (Figure 2A, upper). In addition, the CA-MEKK1 expressing cells significantly increased β-Galactosidase (β-Gal) activity, a marker of cellular senescence (Figure 2A, lower). In order to bypass the senescence and study endogenous effect of CA-MEKK1 on the Arf-p53 pathway, we opted to use transient transfection of the cDNA constructs in wild-type MEFs. Co-expression of Dmp1 and CA-MEKK1 significantly upregulated the Arf, p53, and p21\textsuperscript{Cip1} proteins compared to expression of either construct alone (Figure 2B, left panel). Similarly, co-expression of Dmp1 and CA-MEKK1 in human fibroblasts (IMR90) was more efficient
in upregulating the p14ARF, Hdm2, and p21CIP1 mRNA than either molecule alone. Importantly, Dmp1 and CA-MEKK1 had no effect on the p16INK4A mRNA expression suggesting specificity for the p53 pathway (Supplementary Fig. S2). Since CA-MEKK1 expression in MEFs was a potent inducer of growth arrest and senescence, we established stable MEF clones expressing a Doxycycline-inducible CA-MEKK1. Indeed, doxycycline induction efficiently upregulated the CA-MEKK1 expression and all components of the p53 pathway including Dmp1 (Figure 2B, right panel). In summary, we demonstrate that CA-MEKK1 increases Dmp1 transcriptional function to upregulate the Arf-p53 pathway. The CA-MEKK1 expression alone upregulates the Dmp1-Arf-p53 pathway and induces cellular senescence.

**MEKK1-mediated increase in Dmp1 transcriptional activity is independent of direct Dmp1 phosphorylation.**

In our previous experiments, we observed dependence of the MEKK1 kinase activity to upregulate the Dmp1 transcriptional function. We hypothesized that CA-MEKK1-mediated increase in Dmp1 function is dependent on direct Dmp1 phosphorylation. In the mass spectrometry study of Dmp1 protein with the CA-MEKK1 co-expression, we observed increased abundance of the baseline and additional Dmp1 S/T phosphorylations (Supplementary Fig. S1B, left panel, red and black lettering). Hence, we investigated whether the CA-MEKK1 directly phosphorylates the Dmp1 protein. Using a de-phosphorylation assay with CIP, we demonstrate that CA-MEKK1 and MEKK1-activating drugs, Cisplatin and Toposar, but not the kinase deficient MEKK1 mutant (K1253M), shift Dmp1 protein mobility to a higher phosphorylation state, which is reverted upon CIP treatment (Figure 3A). Furthermore, a recombinant
CA-MEKK1 directly phosphorylated purified Dmp1 protein in an *in vitro* kinase assay (Figure 3B, left panel). The interaction between the Dmp1 and CA-MEKK1 was confirmed with a co-immunoprecipitation experiment in the NIH3T3 cells exogenously expressing both proteins (Figure 3B, right panel).

From the mass spectrometry analysis and prediction algorithms, potential MEKK1 phosphorylation sites on the Dmp1 protein were mapped to 16 S/T residues (Supplementary Figure 1B, left panel). Thus, we chose to narrow down MEKK1-mediated Dmp1 phosphorylation by mutating sections of the Dmp1 protein. Five phospho-deficient Dmp1 mutants were cloned containing 3S/T, 4S/T, 9S/T, 12S/T, and 16S/T mutations to Alanines (A) (Supplementary Fig. S3A). The activity of the newly designed phospho-deficient Dmp1 mutants was tested in the *Arf* promoter activation assay. The wild-type, 3S/T, 9S/T, and 12S/T Dmp1 construct equally activated the *Arf* promoter, while the phospho-deficient Dmp1 mutants, 4S/T and 16S/T, with mutations in the DNA-binding domain were completely inactive (Supplementary Fig. S3B). The phospho-deficient Dmp1 mutations had no effect on Dmp1’s nuclear localization (Supplementary Fig. S4). Next we tested the effect of the phospho-deficient Dmp1 mutations on MEKK1-mediated increase in Dmp1 transcriptional activity. We find that loss of Dmp1 phosphorylation had no effect on the MEKK1-mediated increase in Dmp1 activity (Figure 4). Reduced Dmp1 transcriptional activity was only observed with the 4S/T and 16S/T phospho-deficient Dmp1 mutants, which also had low baseline activity on the *Arf* promoter. Thus, our data supports that MEKK1-mediated increase in Dmp1 activity on the *Arf* promoter is independent of direct phosphorylation.

*MEKK1 increases Dmp1 protein levels independent of transcriptional upregulation.*
Since MEKK1-mediated upregulation of Dmp1 activity on the Arf promoter was independent of direct phosphorylation, we postulated that MEKK1 modulates the Dmp1 protein in a different fashion. In the co-expression experiments with wild-type Dmp1 and the phospho-deficient Dmp1 mutants, CA-MEKK1 increased Dmp1 protein levels regardless of the Dmp1 phosphorylation status and without an effect on the mRNA levels (Supplementary Fig. S5, and data not shown). Likewise, CA-MEKK1 significantly upregulated the endogenous Dmp1 protein while the Dmp1 mRNA was unchanged (Figure 5A). The MEKK1-mediated upregulation of the Dmp1 protein was dependent on the MEKK1 kinase activity since the kinase deficient mutants failed to upregulate the endogenous or exogenous Dmp1 protein (Figure 5A-B). Therefore, MEKK1 kinase activity was necessary for Dmp1 protein upregulation independently of the mRNA modulation.

MAP3K1 locus is frequently inactivated in breast cancer and its expression correlates with patient outcome.

Published work and our unpublished data strongly support 1) a tumor suppressor role of MEKK1 due to frequent inactivation in breast cancer and 2) MEKK1 function in regulating activity of the Dmp1-Arf-p53 tumor suppressor pathway. Therefore, we set out to determine frequency and patterns of the MAP3K1 locus inactivation in human breast tumors. Since MAP3K1 locus was previously reported as a potential tumor suppressor with hemizygous deletions, we utilized LOH microsatellite analysis previously described for the DMP1-INK4A/ARF-p53 pathway (39). The majority of the MEKK1 exons are localized in the 3’ end of the locus; therefore, we identified a four-nucleotide microsatellite repeat outside of the 3’ end and designed a primer set
surrounding the repeats (Figure 6A). The PCR amplification of the microsatellites produced a single band on an agarose gel suggesting that this primer efficiently amplifies the repeats (data not shown). We isolated genomic DNA from 104 patient’s tumors and matched normal breast tissues to determine the qLOH between normal and tumor tissues for the MAP3K1 locus. The qLOH for each patient was calculated by comparing ratio of area under the curve for normal and tumor tissues. A patient was considered LOH positive (LOH+) when qLOH > 2.0 or < 0.5. The representative pattern of hemizygous MAP3K1 deletion (LOH+) in breast cancer is shown in Figure 6A. The qLOH analysis of the MAP3K1 locus in tumor and matched normal tissues from 104 breast cancer patients was found in 21 patients (~20.1%). The reliability of the qLOH analysis for MAP3K1 locus was confirmed with genomic DNA qPCR using a custom TaqMan probe that detects the Exon 14 of MAP3K1 gene (Figure 6B). Although the MAP3K1 loss was not associated with any of the molecular subtypes or stages of breast cancer, its deletion was mutually exclusive with inactivation of the DMP1 locus in 78.9% of the cases ($p < 0.0001, \chi^2 = 21.588$).

Since the MAP3K1 locus was deleted in significant number of breast cancer cases and did not associate with any known subtype of breast cancer, we studied independent prognostic value of MEKK1 expression. Here we utilized a previously published microarray data set in breast cancer to determine effect of differential MEKK1 mRNA expression on the patient outcome (48). The relative tumor MEKK1 expression, detected by two unique microarray probes among the patients, was averaged and the patients were separated into groups with above the mean or below the mean MEKK1 mRNA expression. Based on the MEKK1 expression groups (above and below the mean)
generated with two unique MEKK1 microarray probes, the Kaplan-Meier distant metastasis-free survival curves were graphed. The breast cancer patients expressing MEKK1 above the average had lower probability of distant metastasis compared to the patients expressing MEKK1 below the mean ($p = 0.009$ and $p = 0.019$; Figure 7). In summary, we provide evidence that the $MAP3K1$ locus, which encodes the MEKK1 protein, is hemizygously deleted in ~20% of breast cancer patients and low MEKK1 expression in the tumors was associated with an adverse clinical outcome.

**V.5. Discussion**

Our previous molecular and genetic studies demonstrated that the Dmp1 tumor suppressor function was dependent on activation of the p53 pathway. Overexpression of oncogenes such as mutant Ras or Erbb2 in normal epithelial cells leads to a rapid cell cycle arrest and tumor suppressive senescence, which was mediated by the $Dmp1$ promoter activation. The regulation of the $Dmp1$ promoter was also shown in response to mitogen-stimulated E2F activity. However, the regulation of the Dmp1 protein at a post-translational level has yet to be investigated. In this study, we confirm presence of baseline phosphorylation on multiple Dmp1’s Serine and Threonine residues. Since many transcription factors are regulated by variety of post-translational modifications, detection of Dmp1 phosphorylation suggested that it could be involved in regulating Dmp1 functions. Our mass spectrometry analysis overlapped with algorithmic predictions to show that the SP and TP motifs in Dmp1 protein are most frequently phosphorylated. These motifs are the phosphorylation consensus sequences for kinases
in the MAPK signaling pathway. Hence, our initial findings suggest that Dmp1 may be phosphorylated and post-translationally regulated by members of the MAPK pathway.

In an unrelated experiment, we discovered that a constitutively active member of the MAPK pathway, MEKK1, increased transcriptional activity of Dmp1 on the \(\text{Arf}\) promoter. The observed increase in Dmp1 activity by CA-MEKK1 was dependent on the MEKK1 kinase domain. The MEKK1-mediated increase in Dmp1 activity was recapitulated on the endogenous Arf-p53-p21 pathway. Hence, the MEKK1 kinase domain appears to be a genuine regulator of Dmp1 activity. When we tested the effect of the CA-MEKK1 alone in MEFs, we observed rapid growth arrest and senescence. The MEFs expressing CA-MEKK1 accumulated all components of the p53 pathway including the Dmp1 protein. Thus, our data suggests that the MEKK1 kinase activity signals to increase the Dmp1-Arf-p53 pathway, which blocks cell cycle progression and induces senescence.

Since MEKK1 kinase activity was necessary for increased Dmp1-mediated activation of the \(\text{Arf}\) promoter and since the Dmp1 protein was most commonly phosphorylated on the SP and TP motifs, a phosphorylation motifs of the MAPK signaling kinases, we investigated whether MEKK1 directly phosphorylates the Dmp1 protein to increase its activity. Many studies have confirmed that the endogenous or exogenous Dmp1 protein appear as three bands around \(~120-130\text{kDa}\). We demonstrated that the difference in mobility between the three Dmp1 molecular species was due to a differential abundance of phosphorylation. It was first apparent that Dmp1 could be phosphorylated by MEKK1 when we observed decreased Dmp1 protein mobility in a SDS-PAGE gel with the CA-MEKK1 co-expression. The MEKK1-mediated decrease in
Dmp1 mobility could be reversed with a pan-phosphatase treatment further suggesting that MEKK1 participates in Dmp1 phosphorylation. Importantly, the Dmp1 protein shift was not observed with the kinase deficient MEKK1 mutants. To determine whether MEKK1 can directly phosphorylate Dmp1, we performed an in vitro kinase assay with the recombinant proteins. Indeed, the Dmp1 protein was labeled with P\(^{32}\) when co-incubated with the constitutively active MEKK1. The cellular interaction between Dmp1 and MEKK1 was confirmed by the co-immunoprecipitation. Thus, we provide evidence of MEKK1’s ability to directly phosphorylate Dmp1 and we detect an interaction between these two proteins, which is necessary for the MEKK1-mediated phosphorylation of Dmp1.

Our data strongly suggest the kinase dependent MEKK1 increase in Dmp1 transcriptional activity and that MEKK1 directly phosphorylates the Dmp1 protein. Therefore, we investigated whether an increased Dmp1 activity was a result of direct phosphorylation by MEKK1. In order to connect these two events, we first attempted to identify MEKK1 phosphorylation site on the Dmp1 protein. Using the LC-MS technique, we studied the patterns of Dmp1 phosphorylation in NIH3T3 cells when Dmp1 was expressed alone or with CA-MEKK1. Aside from detecting Serine and Threonine phosphorylation overlap between the two experimental groups, CA-MEKK1 induced phosphorylation on several additional Dmp1 S/T residues. Hence, our data suggests that CA-MEKK1 phosphorylates the Dmp1 protein on multiple S and T residues. In order to identify which MEKK1-phosphorylated S and T residues on the Dmp1 protein contribute to increased Dmp1 activity, we mutated all Serine and Threonine residues identified in the prediction algorithms and mass spectrometry
analysis. Five Dmp1 mutants with up to 16 S/T mutations to Alanines were constructed in segments to investigate the contribution of different domains. When activity of the mutants was tested, it was apparent that S/T mutations in the DNA-binding domain completely blocked Dmp1 transcriptional activity while up to 12S/T mutations in other Dmp1 domains did not abrogate its function. The loss of available phosphorylation sites on Dmp1 was also apparent in the Western blot analyses as the Dmp1 protein with 9, 12, or 16 S/T mutations in the C-terminus appeared as a single band at lower molecular weight. Since phospho-deficient Dmp1 mutants with the mutations in DNA-binding domain displayed the same gel mobility as the wild-type Dmp1 protein, we conclude that the Dmp1 protein is unlikely phosphorylated within the DNA-binding domain. We also confirmed nuclear localization of the Dmp1 mutants since Dmp1 its nuclear localization signal (NLS) has yet to be identified. Hence, our data suggests that baseline transcriptional activity of Dmp1 on the Arf promoter is independent of phosphorylation and that Dmp1 is most likely phosphorylated in the C-terminal transactivation domain.

Findings that Dmp1 phosphorylation has no effect on its baseline transcriptional activity persuaded us to investigate a possibility that the MEKK1-mediated increase in Dmp1 transcriptional activity was due to accumulation of additional phosphorylation on the Dmp1 protein. Using the Arf luciferase assay to study MEKK1-mediated increase in Dmp1 activity, we provide evidence that up to 12 S/T mutations, excluding the mutants with changes in the DNA-binding domain, was as efficient as the wild-type Dmp1 at activating the Arf promoter. The MEKK1-mediated increase in the Arf promoter activity with phospho-deficient mutations in the Dmp1 DNA-binding domain was attributed to the endogenous wild-type Dmp1 protein, which can be upregulated by CA-MEKK1.
Western blot analysis of total protein lysates from this experiment revealed a robust increase in the Dmp1 protein level of all phospho-deficient Dmp1 mutants co-expressing CA-MEKK1. This data further suggests that MEKK1-mediated increase in the Dmp1 activity is due to accumulation of the Dmp1 protein. Indeed, expression of the constitutively active MEKK1, but not the kinase deficient MEKK1, increased the exogenous and endogenous Dmp1 proteins. The Dmp1 mRNA level was unaffected when either the kinase active or deficient MEKK1 was expressed providing an evidence that CA-MEKK1 post-transcriptionally upregulates the Dmp1 protein.

Recent genome-wide analyses of human breast tumors have identified inactivating aberrations of the \textit{MAP3K1} locus. Specifically, the \textit{MAP3K1} was deleted in lung and breast tumor tissues and it was mutated in 8\% of the Luminal A subtype of breast cancer, a group of tumors frequently harboring inactivation of the \textit{DMP1} locus (9-11, 20, 39). The overlap between \textit{DMP1} and \textit{MAP3K1} inactivation in the Luminal A subtype of breast cancer and our data showing that MEKK1 increases the Dmp1 protein evokes a possibility that these two molecules cooperate in breast tumorigenesis. Hence, we investigated frequency of the \textit{MAP3K1} locus deletion using a microsatellite analysis. After analyzing tumor and matched normal breast tissues from 104 patients, the \textit{MAP3K1} locus was found hemizygously deleted in ~20\% of cases. Loss of the \textit{MAP3K1} was mutually exclusive with \textit{DMP1} deletion providing evidence that MEKK1 is a physiological regulator of DMP1 in breast cancer. We verified robustness of the microsatellite analysis with the qPCR to show ~50\% reduction of signal in the tumor compared to matched normal breast tissues when using the samples previously identified as the carriers of hemizygous \textit{MAP3K1} deletion. Since the MEKK1 is an activator of the
Dmp1-Arf-p53 pathway and its locus was inactivated in a significant portion of breast tumors, we speculated that differential MEKK1 expression in tumor tissue could have a biological consequence. Using previously published microarray data sets for human breast tumors, we show that patients expressing a higher level of MEKK1 mRNA had significantly better outcome than the patients with low MEKK1 expression. Thus, our data demonstrate that high MEKK1 expression in the tumors may have a tumor suppressive function, which is in part bypassed with inactivation of the MAP3K1 gene to reduce its expression.

Overall, in this study we demonstrate that even though Dmp1 is heavily phosphorylated, this protein modification does not participate in the Dmp1 transcriptional function. We identify the MEKK1 kinase that directly phosphorylates Dmp1 protein; however, the increase in Dmp1 phosphorylation did not affect its function, but the MEKK1 kinase activity indirectly and post-transcriptionally upregulated the Dmp1 protein. Our preliminary evidence suggests that MEKK1-mediated increase in the Dmp1 protein is independent of known MEKK1 targets (MEK1, JNK1/2, M KK4, and M KK7). The future work will investigate whether MEKK1 stabilizes the Dmp1 protein or signals through translation machinery to accumulate the Dmp1 protein. Even though Dmp1 phosphorylation does not affect its transcriptional function, it is possible that this modification differentially regulates Dmp1 protein interaction with other molecules. Our recent work has shown an Arf-independent function of Dmp1 via direct protein interaction with the p53 (43). Hence, it is possible that phosphorylation and other post-translational modification regulate Dmp1 interacting partners. Furthermore, we show that MAP3K1, the locus encoding MEKK1 protein, is frequently inactivated in breast
tumor cells and its expression correlated with patient outcome. The future investigation will focus on delineating contribution of the \textit{MEKK1} deletion or mutation to drive progression of breast cancer and whether it could be clinically utilized to predict therapeutic response.

\textbf{V.6. Acknowledgments}

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References


Figure 1. Constitutively active MEKK1 increases transcriptional activity of Dmp1. Dmp1 transcriptional activity on the Arf promoter was measured in NIH3T3 using a luciferase reporter. Expression of constitutively active MEKK1 (CA-MEKK1) increased Dmp1-mediated activation of the Arf promoter, which was abrogated with the expression of kinase deficient MEKK1 mutants (K1253M or D1369A).
Figure 2. MEKK1 upregulates the Dmp1-Arf-p53 pathway and induces senescence.

A. Stable expression of CA-MEKK1 in wild-type MEFs induces senescence which was observed by morphologic changes and increased β-Galactosidase (β-Gal) activity. B. CA-MEKK1 increased transcriptional activity of Dmp1 on the endogenous Arf-p53-p21 pathway in wild-type MEFs. Transient co-expression of Dmp1 and CA-MEKK1 upregulates the p19Arf, p53, and p21 expression when compared to the cells expressing either construct alone (left panel). Stable expression of Dox-inducible CA-MEKK1 construct demonstrates upregulation of the p53 pathway upon treatment with 1μg/ml doxycyline for 24 hours (right panel).
Figure 3. MEKK1 directly phosphorylates Dmp1 protein.

A. Treatment of immunoprecipitated Dmp1 with calf-intestinal phosphatase (CIP) or mock to show phosphorylation as a result of various treatments. Dmp1 was phosphorylated by the CA-MEKK1 and MEKK1-activating drugs, Cisplatin and Toposar. The kinase deficient MEKK1 mutant (K1253M) was unable to phosphorylate Dmp1. B. *In vitro* kinase assay with recombinant Dmp1 and CA-MEKK1 proteins shows direct phosphorylation of Dmp1 by CA-MEKK1. CA-MEKK-mediated phosphorylation of MEK1 was used as a control (left panel). Dmp1 and MEKK1 co-immunoprecipitated from NIH3T3 cells indicating their interaction (right panel).
Figure 4. MEKK1-mediated increase in the Dmp1 activity is independent of direct Dmp1 phosphorylation.

Transcriptional activity of the phospho-deficient Dmp1 mutants (3S/T, 4S/T, 9S/T, 12S/T, 16S/T) alone or with CA-MEKK1 co-expressed was compared to wild-type Dmp1 in the Arf promoter luciferase assay. Western blot analysis for each mutant shows protein mobility in the SDS-PAGE indicating loss of phosphorylation. Significant impairment of Dmp1 phosphorylation was observed in Western blot analysis when 9, 12, or 16 Serines and/or Threonines (9S/T, 12S/T, or 16S/T mutants) were mutated in the C-terminus. The Western blot analysis for the exogenous Dmp1 proteins shows longer and shorter exposure.
Figure 5. MEKK1 increases Dmp1 protein independent of transcriptional upregulation.

A. Expression of CA-MEKK1 or kinase deficient MEKK1 mutants (K1253M or D1369) in NIH3T3 cells show MEKK1-mediated upregulation of Dmp1 protein is kinase-dependent. The qRT-PCR analysis demonstrates no significant change in the Dmp1 mRNA upon CA-MEKK1 expression. B. Ectopic expression of Dmp1 with CA-MEKK1 or kinase deficient MEKK1 mutants (K1253M or D1369A) demonstrates increase in Dmp1 protein is dependent on the MEKK1 kinase activity.

- Figure 5.
Figure 6. MAP3K1 locus is hemizygosly deleted in ~20% of human breast tumors.

A. Schematic representation of the MAP3K1 locus on 5q11 chromosome and the location of 6FAM-labeled LOH primers on the 3’ end that amplify a four-nucleotide microsatellite repeat. An example of positive qLOH for MAP3K1 in the tumor compared to matched normal tissue. The qLOH was calculated using following equation: Area Peak 1/Area Peak 2 (normal tissue) divided by Area Peak 1/Area Peak 2 (tumor tissue). Deletion was identified when qLOH >2.0 or <0.5. The arrow indicates the peak that was deleted in the tumor tissue. B. qPCR of 10 tumor and matched normal breast tissues gDNA using a custom designed TaqMan primer in the MAP3K1 Exon 14 to show positive concordance of qLOH analysis with the MAP3K1 locus deletion. Error bars represent experimental variations of the real-time PCR analyses.
Figure 7. MEKK1 expression correlates with distant metastasis-free survival in breast cancer patients.

The MEKK1 (MAP3K1) mRNA expression, detected with two independent probes (AI521181 and AF042838), in breast cancer microarray study were averaged. The patients were segregated into a group that expressed MEKK1 mRNA above the mean versus a group that expressed MEKK1 mRNA below the mean in this study. The Kaplan-Meier distant metastasis-free survival curve was graphed based on the group of patients that expressed MEKK1 above the mean versus the patients expressing MEKK1 below the mean.
Supplementary Figure S1. Dmp1 protein is phosphorylated on multiple Serine and Threonine residues.

A. 1D and 2D gel electrophoresis of immunoprecipitated Dmp1 followed by calf intestinal phosphatase (CIP) treatment demonstrates shift in mobility that indicates a change in protein mass and charge. B. Complete mouse Dmp1 sequence showing predicted MAPK phosphorylation sites (Serine or Threonine followed by Proline) by the mass spectrometry analysis and prediction algorithms. Mass spectrometry of immunoprecipitated Flag-Dmp1 shows baseline level of Dmp1 phosphorylation. Red lettering indicates detected Dmp1 phosphorylation sites (right panel). Many of the identified S/T were also predicted as MAPK phosphorylation sites. Mass spectrometry was also used to identify MEKK1 phosphorylation site(s) on the Dmp1 protein; however, overall increase in phosphorylation of Dmp1 was observed with many overlapping sites identified when Dmp1 is expressed alone. Red and black lettering show sites for Dmp1 phosphorylation when MEKK1 is co-expressed (right panel).
Supplementary Figure S2. Co-expression of Dmp1 and CA-MEKK1 in IMR-90 cells upregulates the ARF-p53-p21<sub>CIP1</sub> pathway.

Transient transfection of Dmp1, CA-MEKK1, or both together in IMR-90 cells followed by the qRT-PCR analysis of p14<sup>ARF</sup>, p21<sup>CIP1</sup>, Hdm2, and p16<sup>INK4A</sup> mRNA expression. Co-expression of CA-MEKK1 and Dmp1 significantly increased all components of the p53 pathway. p16<sup>INK4A</sup> mRNA, a negative regulator of RB pathway, was unchanged.
Supplementary Figure S3. Schematic representation of phospho-deficient Dmp1 mutants and their activity on the Arf promoter.

Supplementary Figure S4. Cellular localization of the phospho-deficient Dmp1 mutants.

Confocal immunofluorescent microscopy of each phospho-deficient Dmp1 mutant in NIH3T3 cells to determine their cellular localization. All of the mutants and wild-type Dmp1 strictly localized in the nucleus. TO-PRO-3 was used to observe nuclear DNA.
Supplementary Figure S5. CA-MEKK1 increases Dmp1 protein level independent of direct Dmp1 phosphorylation.

Co-expression of the phospho-deficient Dmp1 mutants or wild-type Dmp1 with CA-MEKK1 in NIH3T3 cells followed by a Western blot analysis. CA-MEKK1 increased Dmp1 protein level even with 16S/T mutations suggesting a phosphorylation-independent mechanism of Dmp1 protein accumulation.
CHAPTER VI

General Discussion

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This unpublished chapter was composed by Dejan Maglic with an editorial assistance of Guangchao Sui.
VI.1. Her2-Dmp1 interaction in breast cancer

Dmp1 is a 

*bona fide* tumor suppressor whose function is dependent on the Arf-p53-mediated cell cycle arrest and senescence. Generation of the *Dmp1* knockout mouse model revealed that Dmp1 is a critical sensor of replicative senescence. *Dmp1*-null mouse embryonic fibroblasts (MEFs) failed to undergo the p53-mediated growth arrest during serial passaging while maintaining wild-type all components of the p53 pathway. In an *in vivo* setting, *Dmp1* deficient mice were susceptible to variety of tumors during a normal ageing process (1-3). The latency of spontaneous tumorigenesis in the *Dmp1*-null mice was significantly accelerated with exposure to DNA damage-inducing agents suggesting that DNA damage response (DDR) signals to Dmp1 to activate the Arf-p53 pathway (2). Aside from DDR signaling, oncogene-driven hyperproliferative signals upregulate the Dmp1-Arf-p53 activity to prevent early cellular transformation. Specifically, constitutively active Ras mutant activated the *Dmp1* promoter via the Raf-Mek-Erk-Ap1 signaling pathway to ultimately induce p53 (4). Expression of the mutant Ras in *Dmp1*-null bronchiolo-alveolar epithelial cells significantly shortened the latency of lung tumorigenesis (5). Therefore, Dmp1 is an important mediator of replicative and oncogene-induced senescence (OIS) that functions to prevent cellular transformation. Even though Dmp1 was previously described as a *bona fide* tumor suppressor protein, its role during the tumorigenic process of epithelial cells in organs other than lung has not been investigated.

Her2/neu or Erbb2 is a well-described oncogene that drives the tumorigenic process of breast epithelium (6). Yet, Her2 overexpression in normal mammary epithelial cells leads to cell cycle arrest and apoptosis while the hyperproliferating signal
is sustained in the cells with an inactivating $p53$ mutation (7). This observation alluded that the $p53$ pathway is vital in counteracting the Her2-driven tumorigenesis. Her2 is a member of the EGFR family and in breast cancer it activates the Ras-MAPK and PI3K-AKT pathways to drive malignant progression (8). In the second chapter, we reported our investigation on the role of Dmp1 during mammary gland tumorigenesis. First we discovered that Her2, unlike the oncogenic Ras, activates the $Dmp1$ promoter via the PI3K-Akt-NFκB signaling pathway. The Her2-mediated upregulation of Dmp1 led to increase in p53 and its target genes. In the $MMTV$-$Her2/neu$ transgenic mouse model of breast cancer, the $Dmp1$ locus was frequently deleted by the tumor cells to dampen the $p53$-mediated tumor suppression. Similarly, expression of $Her2/neu$ in the $Dmp1$-null mice significantly accelerated mammary tumor development. By analyzing the spontaneously occurring $Dmp1^{+/−}$ tumors, we report that $Dmp1$ is haploinsufficient for tumor suppression. The phenotype of the $Her2/neu$-induced mammary tumors at the $Dmp1^{+/−}$ and $Dmp1^{−/−}$ backgrounds was indistinguishable. The tumors from $Dmp1^{+/−}$ background always retained the other $Dmp1$ allele wild-type supporting the haploinsufficiency model.

Our published data describes the first signaling pathway that connects Her2-induced hyperproliferation and $p53$ activation in the premalignant lesions. Previous research has demonstrated an association between the Her2 expression and $p53$-mediated growth arrest in normal cells; however, the signaling pathway linking these two molecules remains unknown (7). Others have suggested that the ATM-induced DDR signaling participates in senescence activation and tumor suppression (9). Although it is possible that DDR plays an important role in sensing oncogene-induced
hyperproliferation, this mechanism is most likely secondary since the DDR can only be activated following several cycles of replicative stress. Furthermore, previous evidence strongly suggests a direct link between the DDR and Dmp1-mediated activation of the p53 pathway. Indeed, loss of Dmp1 significantly impaired genotoxicity-stimulated activation of the p53 signaling and accelerated tumor development (2, 10). Therefore, it is probable that the DDR also influences Dmp1 activation in the Her2 mammary tumors. Our future research will focus on delineating singling pathways linking the DDR and Dmp1 and understanding their individual contribution during an OIS.

The finding that Her2 activates Dmp1 through the PI3K-Akt-NFκB pathway was surprising since Her2 also activates the MAPK signaling that was previously reported to modulate the Dmp1 promoter (4). This suggests that OIS is regulated by complex signaling networks that are highly dependent on the oncogene and cellular context. In fact, the PI3K-Akt-NFκB pathway is known to promote most stages of malignant process; however, few studies have focused on the role of this pathway during early stages of cell transformation (11). The NFκB family of transcription factors is unique due to ability of its members to heterodimerize with each other and generate differential responses (12). In agreement, previous studies from our lab have shown an inhibitory function of NFκB on the Dmp1 and Arf promoters in response to anthracyclines (13). Prospective and more detailed analysis of the NFκB subunits binding to the Dmp1 promoter and their interaction with other chromatin modulators will reveal a better mechanistic understanding of how upstream signaling pathways affect the NFκB activator or repressor functions.
VI.2. The DMP1 role in human breast cancer

Development of the high-throughput microarray analyses has been instrumental in molecular characterization of breast tumors into five new subcategories. The breast cancers were sub-grouped based on the expression profiles that closely associated with the existing hormone/growth factor receptor markers in combination with the proliferation index (14). Importantly, the new breast cancer subtypes predicted clinical response to the existing radiotherapy and chemotherapeutic regiments (15-16). Distinct responses to genotoxic agents between the subtypes were attributed to the mutation status of \( p53 \), a sensor of DNA damage and regulator of apoptotic response (17). The breast cancer patients with the most adverse clinical outcome bear the highest rate of the \( p53 \) inactivation while the subtype carrying lowest incidence in \( p53 \) mutation has the best clinical outcome (18). Observed differential \( p53 \) status among the breast cancer subtypes suggests that there is an existence of variable selective pressures to inactivate \( p53 \) during tumor progression.

Dmp1 is an essential modulator of the Arf-p53 pathway in variety of tissues including mouse mammary gland. In the second chapter, we provide strong evidence that Dmp1 is a critical link between Her2 and the p53-mediated OIS (19). Our \textit{in vitro} data was recapitulated in the \textit{in vivo} mouse model of breast cancer to show that the p53 tumor suppression in premalignant lesions was dependent on activity of the Dmp1-Arf axis. By studying tumor progression in this model, we revealed an OIS escape mechanism that ensues with hemizygous \( Dmp1 \) deletion to dampen the Arf-p53 pathway and relieve the cell cycle arrest (19). The human DMP1 protein is encoded from chromosome 7q21, a locus frequently inactivated in variety of tumor types (20-22). In lung cancer, deletion of
the DMP1 locus altered the spectrum of INK4A/ARF and p53 inactivation (5). Therefore, we hypothesized that alteration of the DMP1 locus in breast cancer could change the pattern of p53 inactivation that we observe among the breast cancer subtypes. With the analysis of DMP1-ARF-Hdm2-p53 pathway inactivation in a large cohort of breast cancer patients, we discovered that the DMP1 locus is hemizygosly deleted in up to 42% of breast tumors. Importantly, loss of DMP1 was mutually exclusive of the p53 gene deletion or mutation. This finding was associated with clinical significance as the patients with DMP1 deletion (maintaining wild-type p53) had significantly better outcome than the patients whose tumors retained both DMP1 alleles. In agreement with the established paradigm, DMP1 deletion was exclusively associated with the Luminal A subtype of breast cancer, which is a group with the lowest incidence of p53 inactivation and best clinical outcome. Our detailed mapping of the DMP1 locus also demonstrates high specificity for DMP1 gene deletion suggesting that this process is not stochastic as believed for many other chromosomal aberrations. Although very intriguing question in cancer biology, it remains unknown whether cancer cells have a capacity to engage precise mechanisms and focally delete certain genomic regions. Since the MMTV-Her2 tumors also delete one Dmp1 allele, this model could be used in the future studies to identify factors during cell division that promote Dmp1 locus deletion.

Our findings in the third chapter offer multiple novel implications and provide for better understanding of breast cancer biology. First, we demonstrate that DMP1 is a physiological regulator of the ARF-p53 pathway in mammary epithelium. Similar to the results seen with the mouse model of breast cancer, the human DMP1 gene is inactivated in order to dampen the p53-mediated OIS (19). Since the tumors bearing DMP1 deletion
kept the p53 locus wild-type, it is now clear that this is one of the major OIS escape mechanisms in breast cancer. Furthermore, it explains diversity in the p53 status among the breast cancer subtypes. We also studied the correlation between HER2 and DMP1 expression in tumors to show that the HER2 oncogene, like in the mouse model, is one of the DMP1 activators. However, DMP1 deletion did not correlate with the HER2 expression revealing that other signaling pathways may regulate DMP1 activity during tumorigenesis. While very few oncogenic aberrations are known to drive the Luminal A subtype of breast cancer, a group most commonly associated with DMP1 deletion, it is possible that indirect DDR signaling regulates the DMP1 tumor suppressor pathway. Indeed, the Dmp1 knockout mouse model has previously demonstrated an important role of Dmp1 in DDR-dependent tumor suppression (2-3). The future research will need to delineate the crosstalk between DDR and DMP1 in order to determine their epistatic regulation.

VI.3. Alternative splicing of DMP1 in breast cancer

The mouse and human data strongly support DMP1 role in activating the p53-mediated OIS to prevent breast carcinogenesis. The progression of cancer cells is associated with hemizygous DMP1 deletion or biallelic p53 inactivation to bypass the OIS (23). Although the haploinsufficiency of Dmp1 for tumor suppression was demonstrated in multiple mouse models and human tumors, it remained uncertain why loss of single Dmp1 allele recapitulated the phenotype observed in the mice with biallelic Dmp1 deletion (3, 5, 19). The mechanism of haploid insufficiency of Dmp1 and other haploinsufficient tumor suppressors has not been investigated in great detail. In case of
Dmp1, a mere 50% reduction in the protein expression does not explain the phenotypic parallel seen in the $Dmp1^{+/}$ and $Dmp1^{-/-}$ animals. Others have also suggested contribution of additional post-transcriptional and post-translational mechanisms that inactivate the intact alleles of haploinsufficient genes.

The observation that $DMP1$ inactivation in the tumors resulted in only one allele loss in more than 99% of the cases analyzed suggests that other mechanisms could contribute to inactivation of the remaining $DMP1$ allele (23). Since the intact $DMP1$ allele lacked inactivating mutations or promoter hypermethylation, we hypothesized that aberrant post-transcriptional mechanisms were likely accountable for further reducing DMP1 expression. Recent evidence suggested that human $DMP1$ locus encodes at least three unique DMP1 isoforms via alternative splicing of the $Exon\ 10$ (24). The bona fide tumor suppressor and transcription factor was named as DMP1α, while two new DMP1 isoforms lacking canonical DMP1α functions were named as DMP1β and DMP1γ. Since many of the latest studies have implicated aberrant splicing as a driver of tumor progression, we investigated the function of DMP1 splice isoforms in breast cancer (25). By studying cell lines and clinical samples, we demonstrated that the $DMP1$ locus is aberrantly spliced in the tumor cells to increase the DMP1β to DMP1α mRNA ratios. The aberrant splicing in the tumor tissues was associated with adverse breast cancer patient outcome. Importantly, the aberrant splicing of DMP1 occurred in the breast cancers carrying hemizygous $DMP1$ deletion and in the tumors with wild-type $DMP1$. These data signify that the DMP1 alternative splicing could be utilized by the cancer cells to inactivate remaining $DMP1$ alleles and contribute to the mechanism of haploinsufficiency.
In order to investigate opposing roles of DMP1 isoforms in mammary gland biology, we established a novel transgenic mouse model expressing DMP1β in the mammary epithelium. Whereas the published work shows that overexpression of DMP1α in the mammary gland blocks proliferation of the epithelial cells, DMP1β in the same setting induced diffuse hyperplasia and palpable mammary gland tumors (26). Thus, the in vivo data clearly demonstrated unique roles of the DMP1 isoforms in the mammary epithelium and confirms that aberrant DMP1 splicing is an important driver of malignant progression. Notably, DMP1β’s effect in breast cancer was independent of the DMP1α-ARF-p53 tumor suppressor pathway. Since the DMP1β protein lacks the DNA-binding and transactivation domains found in DMP1α, most likely it exerts its function through physical interactions with other cellular molecules. Prospective studies will need to identify DMP1β binding partners to mechanistically understand its tumor-promoting functions. Furthermore, upcoming studies should be conducted to elucidate a functional difference between the DMP1β and DMP1γ isoforms and delineate how an additional 14 amino acids in the DMP1γ C-terminus contributes to its distinctive function.

Contribution of DMP1 splicing and its role in tumorigenesis have not been investigated in other tumor types that display DMP1 haploinsufficiency. In addition, it is unknown how the alternative splicing of DMP1 participates in the normal mammary gland physiology or biology of other tissues. Current evidence supports that the Dmp1 locus could participate in functions other than tumor suppression. Dmp1−/−, but not the Dmp1+/−, male and female mice display a significant growth retardation with up to 30% reduced body mass. Moreover, mammary glands from the Dmp1−/− females fail to properly differentiate during pregnancy (2). Detailed analysis of the Dmp1−/− mammary
glands demonstrated impairment in the luminal cell proliferation whose function is necessary for milk production. Therefore, the haploid insufficiency of *Dmp1* was only found in the context of tumor development but not with other physiological functions. Future investigations of developmental stages in the *Dmp1<sup>+/−</sup>* and *Dmp1<sup>−/−</sup>* mice could have a key role in elucidating mechanistic functions of each DMP1 isoform and reveal the tumor’s propensity to aberrantly splice *DMP1* locus.

**VI.4. Dmp1 regulation by MEKK1 and its role in breast cancer**

Extensive molecular analysis of the Dmp1 and OIS interplay provided evidence that the Dmp1 activity is modulated via the promoter activation or repression (4, 19, 27). To date, no studies have found evidence of transcription-independent Dmp1 regulation. Based on amino acid sequence, the Dmp1 protein is predicted to have a molecular size of ~85kDa; however, exogenous or endogenous Dmp1 protein is always observed around ~120-130kDa (28). In a SDS-PAGE gel, the Dmp1 protein most commonly appears as three bands indicating multiple molecular species. Hence, Dmp1 appears to be a highly modified protein. Series of algorithms have predicted the Dmp1 protein sequence to be decorated by many post-translational modifications including ubiquitination, phosphorylation, sumoylation, and glycosylation. The function of any of these Dmp1 modifications has not been examined. In the literature, functional roles of post-translational modification have been described for variety of transcription factors (29). Since Dmp1 is a highly modified protein, we hypothesized that its activity could be modulated at the post-translational level.
The first evidence that the Dmp1 protein is modified via phosphorylation was observed during the protein-protein interaction experiments with purified Cdk4/cyclin D complex (28, 30). The function of Dmp1 phosphorylation in this setting was never further pursued. On the other hand, our preliminary experiments demonstrated that Dmp1 protein, regardless of cell cycle phase, has a relatively long half-life with up to 8 hours. This suggested that the ubiquitination and proteasomal degradation system plays a minimal role in regulating the Dmp1 protein turnover. Therefore, we decided to focus on understanding Dmp1 phosphorylation and its effects on Dmp1 activity. Using in vitro and cell expression systems described in the fifth chapter, we confirm extensive Dmp1 phosphorylation on the SP and TP motifs and discover a kinase, MEKK1, which directly phosphorylates the Dmp1 protein. Despite extensive phosphorylation, Dmp1 transcriptional activity remained unaffected. The observed MEKK1-mediated increase in Dmp1 activity was dependent on post-transcriptional accumulation of the Dmp1 protein. For the first time, we discovered a Dmp1 modulator that upregulates the Dmp1 protein independent of its promoter activation.

In order to identify the MEKK1 phosphorylation site on the Dmp1 protein, we generated multiple phospho-deficient Dmp1 mutants, some of which displayed distinct protein mobility in the Western blot analysis. Specifically, mutations in the C-terminal domain of Dmp1 increased protein mobility in a SDS-PAGE gel indicating that this domain is the site of most Dmp1 phosphorylations. However, the phospho-deficient Dmp1 mutants that show complete absence of phosphorylation retained transcriptional activity equal to the wild-type Dmp1. This finding supported our conclusion that Dmp1 transcriptional activity was independent of phosphorylation. The observation that Dmp1
protein is highly decorated with energy dependent post-translational modifications like phosphorylation conveyed to us that they may be important for Dmp1 functions aside from transcription. Indeed, we have recently reported transcription-independent role of Dmp1 during direct interaction with the p53 protein (10). Therefore, our next efforts will explore effect of Dmp1 phosphorylation and other modifications on Dmp1’s physical interaction with other molecules.

With the identification of Serine/Threonine kinase, MEKK1, a novel regulator of Dmp1 function, we began unlocking how MEKK1 participates in breast tumorigenesis (31). Multiple sequencing analyses of breast tumors have recently reported frequent inactivating mutations in the MEKK1 kinase domain (32-34). Currently, no mechanistic studies have described how MEKK1 functions as a tumor suppressor gene. One possible reason is that the 195kDa molecular size of MEKK1 protein prevents a quick in vitro manipulation. In addition, the MEKK1 protein is composed of N-terminal regulatory and C-terminal kinase domains (35). Since mutations were most commonly found in the MEKK1 kinase domain, it is most likely that the kinase activity is a functional unit of this protein. Hence, in our studies we used the kinase active MEKK1 constructs to investigate its cellular function. We discover that constitutively active MEKK1 increases abundance of Dmp1 protein and all other components of the p53 pathway to activate a senescence program. Thus, it is possible that tumor suppressor function of the MEKK1 kinase domain is dependent on the Dmp1-Arf-p53 signaling pathway. The future studies should focus on identification of physiological MEKK1 activators that signal to the Dmp1-Arf-p53 pathway in normal and cancer cells.
The analyses of MAP3K1 aberrations have indicated that the MAP3K1 mutations exclusively clustered in the Luminal A subtype of breast cancer, a group of patients that frequently carry DMP1 deletions (32-34). Observed commonality of the MAP3K1 and DMP1 aberrations in a particular group of breast tumors further supported an idea that these two molecules share functional similarity. Aside from mutation, comparative genome hybridization (CGH) analyses of human lung and breast tumors revealed that up to 18% of tumors deleted one MAP3K1 allele (36). Based on this evidence, we analyzed the MAP3K1 deletion pattern in breast cancers using a PCR-based microsatellite analysis. We observed hemizygous MAP3K1 deletion in ~20% of breast tumors confirming that MEKK1 is an important tumor suppressor in breast cancer. The MAP3K1 deletion did not associate with any one subtype of breast cancer but it was mutually exclusive with DMP1 deletion. This finding suggests that DMP1 and MEKK1 co-regulate each other in human breast cancer. Next, we studied the prognostic value of MEKK1 expression and found that low MEKK1 mRNA in the breast tumors was associated with adverse patient outcome. Overall, our data provide evidence of existing selective pressure to inactivate the tumor suppressive function of MAP3K1 gene, which ultimately predicts poor clinical outcome. Our future efforts will be focused on sequencing the MAP3K1 gene in combination with the LOH analysis to understand the overlap between these two events in breast cancer. In addition, novel mouse models with inactivating MEKK1 mutations or gene deletion will be established to study their role in mammary tumor initiation and contribution to a therapeutic resistance.

VI.5. Conclusions
Our data, presented in this dissertation, support an integral role of mouse and human Dmp1 protein in preventing breast tumorigenesis (Figure 1). During tumor initiation, Her2 overexpression activates the PI3K-AKT-NFkB pathway which signals to the Dmp1 promoter to induce its expression. The Dmp1 protein binds to the Arf promoter to increase Arf expression, which functions to block the Mdm2-mediated degradation of p53. The p53 exerts its tumor suppressor function through activation of numerous cell cycle or apoptotic regulators. Thus, oncogenic transformation of mammary epithelium is averted by the Dmp1-Arf-p53 signaling pathway that activates a cell cycle arrest and senescence. However, in the first step of tumor initiation, certain epithelial cells escape the tumor suppressive mechanism and gain a malignant capacity. These cells delete the DMP1, INK4A/ARF, or p53 locus in a mutually exclusive fashion to allow cell cycle progression and proliferation of the transformed cells. In the second step of malignant progression, breast tumor cells alter the splicing machinery to aberrantly splice the DMP1 locus to further reduce expression of the tumor suppressor DMP1α and increase expression of the tumor-promoting DMP1β isoform. The alternative DMP1 splicing contributes to inactivation of its tumor suppressor function in the patients’ tumors with hemizygously deleted or wild-type DMP1 locus supporting that aberrant splicing is a novel mechanism contributing to the DMP1 haploinsufficiency.
Figure 1: Model of DMP1-ARF-p53 pathway modulation during breast cancer initiation and progression:

**Tumor initiation**
- **Her2**: Amplification ~20%
- **PI3K**
- **AKT**
- **NF-κB**
- **MEKK1**: ~20%
- **ARF**: Amplification ~13%
- **Hdm2**
- **p53**: ~34% Growth Arrest Senescence

**Tumor progression**
- **MYC**
- **YB1**
- **Her2**
- **hnRNP SRSF**
- **DMP1α** ~42%
- **DMP1β**
- **Overexpression ** ~30%
- **Proliferation**

= oncogene
= tumor suppressor
= gene deletion
= gene amplification and/or overexpression
References


Curriculum Vitae

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Education

Doctorate of Philosophy
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Honors and Awards

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Dejan Maglic, Robert Kendig, Elizabeth Fry, and Kazushi Inoue. DMP1β, an alternative splice isoform of tumor suppressor hDMP1 locus, has oncogenic properties in breast cancer. 109th Annual Meeting North Carolina Academy of Science, Buies Creek NC, 2012.

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**Leadership Positions**

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Professional Affiliations

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