

# **m6A's Unique and Dynamic Role in Breast Cancer Progression**

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## **Abstract**

Despite intense study, metastatic breast cancer is still the second leading cause of female death from cancer in the US. While many genetic lesions and environmental factors have been implicated in breast cancer progression, effective treatments are still lacking, suggesting that we are missing part of the puzzle. Epithelial to Mesenchymal Transition (EMT) is a genetic process by which breast cancer cells epithelial characteristics are changed into a more invasive and mobile mesenchymal phenotype through the downregulation of epithelial genes and pathways while simultaneously upregulating mesenchymal genes and pathways. In recent years, it has become clear that posttranscriptional regulation plays a key role in the aberrant gene expression underlying malignancy and metastasis. For example, the mRNA modification N6-methyladenosine (m6A) is involved in many post-transcriptional regulation processes including mRNA stability and translational efficiency and has been reported to be involved in many different cancer types, including breast cancer. Unfortunately, there are many characterizations of m6A's role in not just breast cancer, but all types of cancers that are often conflicting. For this study, we characterized the effects of decreasing mRNA m6A levels by knocking down METTL3 in the MCF10 genetically defined model of breast cancer. The goal of this study was to determine if effects on proliferation and migration differed based on the stage of disease progression. Here we report that

knocking down METTL3 at distinct stages of breast cancer progression indeed shows unique effects at each stage. The early-stage breast cancer line showed a more proliferative phenotype with the knockdown of METTL3 while the transformed breast cancer line showed a more migratory phenotype. Interestingly, the metastasized breast cancer cell line showed almost no effect on phenotype with the knockdown of METTL3. Furthermore, our transcriptome wide analysis of these cell lines with the knockdown of METTL3 provided us with a possible mechanism of m6A regulating EMT thus resulting in these phenotypical changes we observed. Finally, this study may begin to address the controversy of m6A's role in cancer and suggests that m6A may have a dynamic role in cancer that depends on the stage of progression.



# **m6A's Unique and Dynamic Role in Breast Cancer Progression**

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# Table of Contents

List of Figures .....	v
Abbreviations .....	vi
Chapter 1: Literature Review .....	1
Cancer, its discovery, and its diversity. ....	2
Stages of Cancer .....	4
Breast Cancer .....	7
Epithelial to Mesenchymal transition. ....	9
EMT genes.....	10
m6A.....	13
m6A and Cancer .....	18
m6A and EMT targets.....	21
Conclusion.....	23
References.....	25
Chapter 2: m6A and its Relationship with Breast Cancer Progression .....	40
Introduction .....	41
Results.....	44
Characterization of the MCF10 Breast Cancer Model.....	44
Knocking Down METTL3 and its effect on phenotype .....	46
Changes in the transcriptome with the knockdown of METTL3 .....	48
Overlap of gene changes within the MCF10 Breast Cancer model with the Knockdown of METTL3 .....	50
M6A levels with the knockdown of METTL3 in Breast Cancer .....	50
EMT gene expression .....	53
Discussion.....	55
Methods.....	61
Appendices.....	66
References.....	71
Chapter 3: Discussion.....	75
Introduction .....	76
Justifications.....	76



MCF10 model .....	76
Development of the METTL3 Knockdown Cell lines .....	77
Phenotypic Assays .....	78
Contributions to the field.....	79
Future Directions.....	82
Clinical Relevance.....	88
Conclusion.....	89
References.....	91

## List of Figures

<b>Chapter 1: Literature Review .....</b>	<b>1</b>
<b>Figure 1.1: Hallmarks of Cancer. ....</b>	<b>3</b>
<b>Figure 1.2: Model of Linear and Branching Cancer Progression. ....</b>	<b>5</b>
<b>Figure 1.3: EMT-MET Loop with Key Players. ....</b>	<b>11</b>
<b>Figure 1.4: The METTL3- METTL14-WTAP Complex. ....</b>	<b>14</b>
<b>Figure 1.5: m6A, Writers, Erasers and Readers. ....</b>	<b>17</b>
<b>Figure 1.6: m6A and its Currently Understood Role in Cancer. ....</b>	<b>18</b>
<b>Chapter 2: m6A and its Relationship with Breast Cancer Progression .....</b>	<b>40</b>
<b>Figure 2.1: The MCF10 Breast Cancer Model. ....</b>	<b>45</b>
<b>Figure 2.2: m6A and its effect on phenotype. ....</b>	<b>49</b>
<b>Figure 2.3: Transcriptomics of METTL3 Knockdown in the MCF10 Breast Cancer Model. ....</b>	<b>52</b>
<b>Figure 2.4: mRNA and Protein Expression Changes with the Knockdown of METTL3 in the MCF10 Breast Cancer Model. ....</b>	<b>54</b>
<b>Appendix A: First FACS of Neg Crispr and METTL3 Knockdown in the MCF10Ca1H Lines .....</b>	<b>66-67</b>
<b>Appendix B: Second FACS of Neg Crispr and METTL3 Knockdown in the MCF10Ca1H Lines .....</b>	<b>69-69</b>
<b>Appendix C: Western Blot of Caspase 9 in all three cell lines with METTL3 Knockdown .....</b>	<b>70</b>

## Abbreviations

AKT	-	alpha serine/threonine-protein kinase
ALKBH5	-	AlkB Homolog 5, RNA Demethylase
AML	-	Acute myeloid leukemia
AXL	-	AXL receptor tyrosine kinase
BPTF	-	Bromodomain PHD Finger Transcription Factor
BRCA1	-	Breast Cancer Associated Gene 1
BRCA2	-	Breast Cancer Associated Gene 2
Cas9	-	CRISPR-Associated Protein 9
CDK	-	Cyclin-dependent kinase
circRNA	-	Circular Ribonucleic Acid
c-MYC	-	Cellular Myelocytomatosis Oncogene
CRISPR	-	Clustered Regularly Interspaced Short Palindromic Repeats
DMEM	-	Dulbecco's Modified Eagle Medium
DPBS	-	Dulbecco's Phosphate Buffered Saline
ECM	-	Extracellular Matrix
ECS	-	Extracellular Structure
EGFR	-	Epidermal Growth Factor Receptor
EIF3	-	Eukaryotic Initiation Factor 3
EIF4A3	-	Eukaryotic Translation Initiation Factor 4A3
EMT	-	Epithelial to Mesenchymal Transition
ENO1	-	Alpha Enolase

ER+	-	Estrogen Receptor Positive
f6A	-	N6-formyladenosine
FACS	-	Fluorescence-Activated Cell Sorting
FOXC2	-	Forkhead Box Protein C2
FOXO1	-	Forkhead Box Protein O1
FTO	-	Fat Mass and Obesity Associated
GLUT1	-	Glucose Transporter Protein Type 1
HEC-1a	-	Human Endometrial Adenocarcinoma 1a
HeLa	-	Immortalized Cervical Cancer Cell Line named after HEnrietta LACKs
HepG2	-	Human Hepatocellular Carcinoma G2
HK1	-	Hexokinase 1
HK2	-	Hexokinase 2
hm6A	-	N6-hydroxymethyladenosine
HNSCC	-	Head and Neck Squamous Cell Carcinoma
HR+	-	Hormone Receptor Positive
LEF-1	-	Lymphoid Enhancer Binding Factor 1
LH	-	Leader Helix
lncRNA	-	Long Non-Coding Ribonucleic Acid
LUSC	-	Lung Squamous Cell Carcinoma
m6A	-	N6-Methyladenosine
m6Am	-	N6,2'-O-dimethyladenosine
MCF10	-	Michigan Cancer Foundaion-10
MET	-	Mesenchymal to Epithelial Transition
METTL14	-	Methyltransferase-14

METTL16	-	Methyltransferase-16
METTL3	-	Methyltransferase-3
miRNA	-	Micro Ribonucleic Acid
MM	-	Multiple Myeloma
MMP2	-	Matrix Metalloproteinase 2
MMP9	-	Matrix Metalloproteinase 9
MPTS	-	p-methoxyphenyl p-toluenesulfonate
mRNA	-	Messenger Ribonucleic Acid
mTORC1	-	Mammalian Target of Rapamycin Complex
Mucin1	-	Mucin Short Variant S1
MZF1	-	Myeloid Zinc Finger 1
NCI	-	National Cancer Institute
NF- $\kappa$ B	-	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NLS	-	Nuclear Localization Signal
p27	-	Cyclin-dependent Kinase Inhibitor 1B
p53	-	Tumor Protein P53
PGC-1 $\alpha$	-	Peroxisome Proliferator-Activated Receptor Coactivator-1alpha
PR+	-	Progesterone Receptor Positive
PTEN	-	Phosphatase and Tensin Homolog
RGG	-	Arginine-Glycine Repeats
RKIP	-	Raf Kinase Inhibitory Protein
RNA	-	Ribonucleic Acid
rRNA	-	Ribosomal Ribonucleic Acid
RT-PCR	-	Real Time Polymerase Chain Reaction

RUNX1	-	Runt-Related Transcription Factor 1
SAM	-	S-Adenosyl-L-methionine
shRNA	-	Small Hairpin RNA
Snail	-	Snail Family Transcriptional Repressor 1
snRNA	-	Small Nuclear Ribonucleic Acid
SPI1	-	Spi-1 Proto-Oncogene
SRSF3	-	Serine and Arginine Rich Splicing Factor 3
SRSF10	-	Serine and Arginine Rich Splicing Factor 10
TAZ	-	Phospholipid-Lysophospholipid Transacylase
TBST	-	Tris Buffered Saline with Tween 20
TGF- $\beta$	-	Transforming Growth Factor Beta-1
TMN	-	Tumor Size-Lymph Nodes-Metastasized
TRCA	-	T-cell receptor alpha locus
TrmM	-	Triple-Refractory Multiple Myeloma
VEGF	-	Vascular Endothelial Growth Factor
WCEB	-	Whole Cell Extract Buffer
Wnt	-	Wingless/Integrated Pathway
WTAP	-	WT1 Associated Protein
YTHDC1	-	YTH N6-Methyladenosine RNA Binding Protein C1
YTHDF1	-	YTH N6-Methyladenosine RNA Binding Protein F1
YTHDF2	-	YTH N6-Methyladenosine RNA Binding Protein F2
YTHDF3	-	YTH N6-Methyladenosine RNA Binding Protein F3
ZEB-1	-	Zinc Finger E-Box Binding Homeobox 1

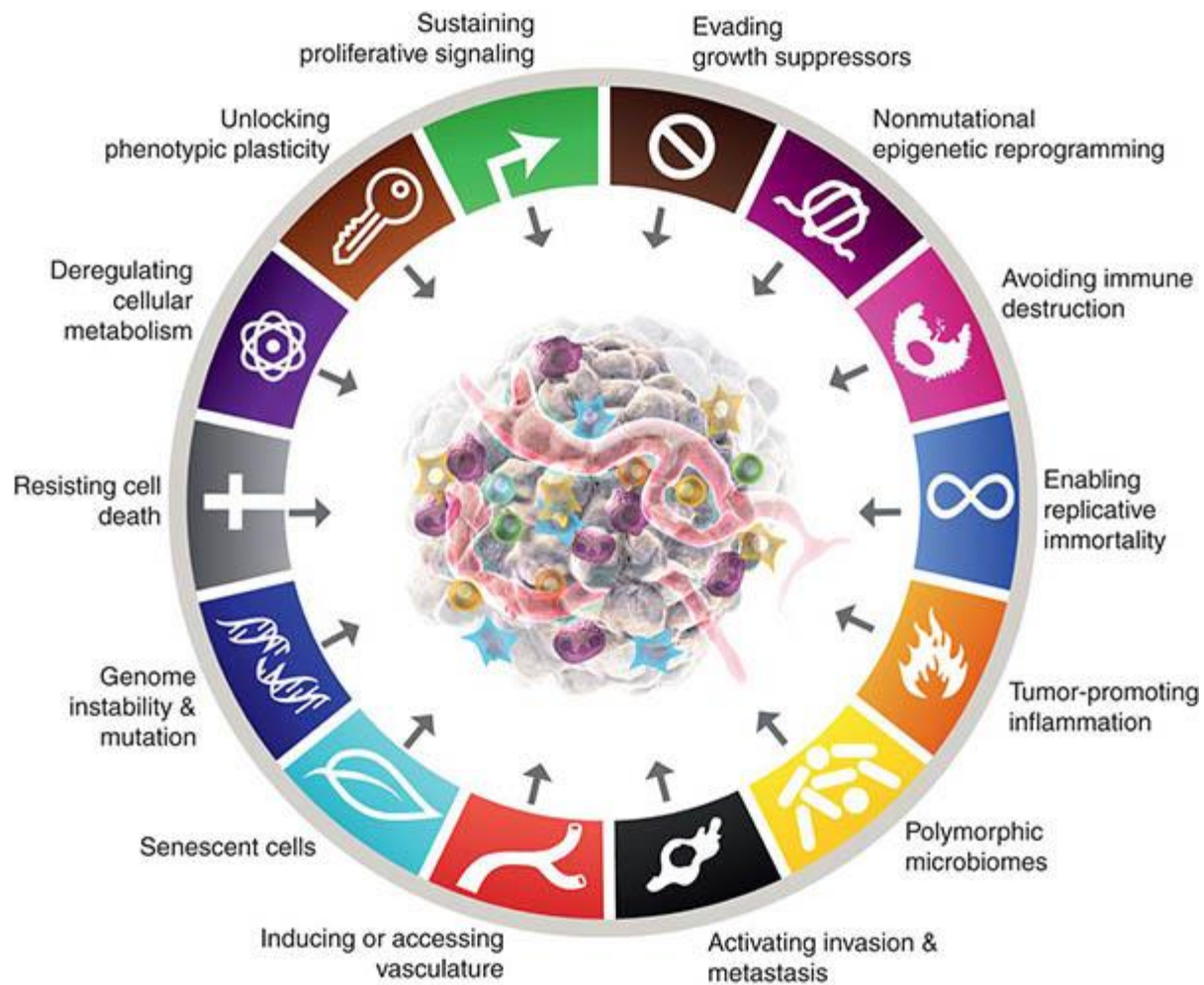
# **Chapter 1: Literature Review**

## **Cancer, its discovery, and its diversity.**

Cancer is a disease in which cells begin proliferating uncontrollably and become immortalized, no longer following the Hayflick Limit. The Hayflick Limit states that cells can replicate between forty and sixty times. The first recorded occurrence of cancer in medical manuscripts was discovered in the 19<sup>th</sup> century in Egyptian papyri written in 1600 B.C. Others suggest the first attempts at understanding cancer date back 4500 years ago [1]. Since then, many discoveries have been made about cancer such as progression events that occur after immortalization. One of the largest discoveries in the cancer field is the Warburg effect [2]. Otto Warburg in the 1920's had observed that tumors were consuming much higher levels of glucose compared to neighboring tissue [3]. He also observed that these cancer cells had the capability of fermenting the glucose in the presence of oxygen leading to the term aerobic glycolysis [4]. This single trait has been the subject of study for almost one hundred years with thousands of journal articles published in this regard [5]. As of 2022 there are fourteen hallmarks of cancer such as sustaining proliferative signaling, resisting cell death, genomic instability and mutation, activation invasion and metastasis as well as others seen in **Figure 1.1** [6]. Not all cancers show all fourteen hallmarks and not all cancers show the same combination of these hallmarks.

Cancers are often times categorized into two groups (solid and liquid). Solid cancers, also known as organ cancers, are as the name suggests. They are cancers that begin in organ tissue and if left unchecked, invade neighboring tissues as well as satellite sites. Liquid cancers are commonly known as blood cancers found in the bodily fluids and are in constant suspension.





**Figure 1.1: Hallmarks of Cancer.** There are 14 capabilities and characteristics found in cancer that are both topics of study as well as potential therapeutic targets in treating different cancers. Image taken from Hanahan D [125].

Not only do cancers vary based on location in the body, but also vary in the mutations that allow for the cancer to obtain one or more of the fourteen hallmarks of cancer. Some mutations are very common in cancer such as the transcription factor *p53*. *p53* is a tumor suppressor gene that will induce cellular responses. One of these responses is cell cycle arrest that occurs due to several cell signals such as DNA damage and oncogene damage. If the DNA damage is not repaired *p53* will recruit machinery that will lead to apoptosis for cells. *p53* is mutated in approximately half of all cancers [43]. Other mutations are

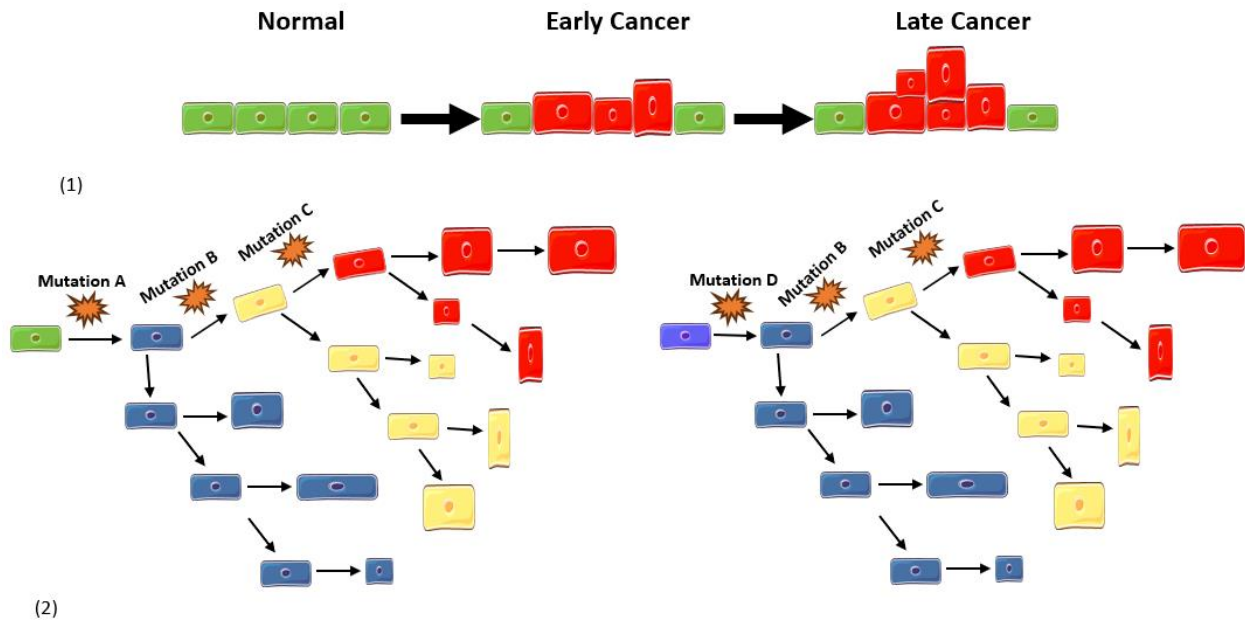
more specific to the cancer type such as the transcription factors Breast Cancer Type 1 Susceptibility protein (*BRCA1*) and Breast Cancer Type 2 Susceptibility protein (*BRCA2*). Like p53, *BRCA1/2* are also tumor suppressor genes that as their names suggest, are more commonly found in breast and ovarian cancers compared to others and having these mutations significantly increases cancer risk [44]. Though there are certain target genes used for diagnosing as well as determining risk levels of cancer, rarely is a single mutation the sole cause of cancer progressing through its stages.

Cancer is a dynamic disease that is constantly adapting to its environment, whether it be neighboring tissue, hypoxia, nutrient level, etc. These adaptations occur via subsequent mutations that allow the cancer to develop characteristics as seen in **Figure 1.1**. It should also be noted that cancer mutations are not linear but rather branch leading to a remarkably diverse set of cells within a tumor which is modelled simplistically in **Figure 1.2**. Depending on which mutations occur, new lineages produce different subpopulations within a tumor. This diversity within a tumor is known as tumor heterogeneity where a tumor can have several subpopulations of cells that differ in mutations present. Since cancer tumors no longer scaffold properly onto the extra cellular matrix, they have differing environments in several regions that influence the cell in order to survive in those conditions. These environments could be necrotic, hypoxic, mitotic, as well as others. Cancer's high adaptability leads to tumor heterogeneity and also makes some cancer much harder to treat. What can potentially occur is that treatment affects the majority of the cells in a tumor while the remaining cells become resistant to the treatment. The cancer cells that survive the treatment progress further into the later stages. Potentially a cocktail of cancer drugs can be produced to address this heterogeneity in order to treat several subpopulations simultaneously as well as ensuring the cancer cells are unable to become resistant to the several drugs within the cocktail.

## **Stages of Cancer**

Clinically there are five stages of cancer, 0 through IV, that can be determined by a pathologist. Stage 0 also known as "in situ" meaning the cancer has not changed its place of formation. Surgery can sometimes remove this initial tumor in its entirety and thus curing the patient. Stage I is defined as the

tumor beginning to grow but maintaining its in situ characteristic. Stage II is defined when the tumor reaches an even larger size but has not spread to neighboring tissues and lymph nodes. The stage III label is given to cancers that have begun to spread to neighboring tissues as well as proximal lymph nodes. The final stage, Stage IV, is determined once the cancer has spread to satellite sites away from the primary tumor site.



**Figure 1.2: Model of Linear and Branching Cancer Progression.** Cancer Progression is generally depicted in a linear or branching model. The linear model (A) is used in order for presenting differences in stages of cancer as well as simplicity. The (B) branched model is utilized in order to present the diversity of subpopulations that can occur based on different mutations that can be observed within a tumor. Each color denotes a mutation that is not present in the previous color and each branch shows the lineage of subpopulation. Image taken and modified from Hendricks et al [126].

There are some considerations to take when determining stage of a cancer biopsy or diagnosis. The first is that as mentioned previously, cancer cells in tumors do not progress in synchronization with one another. Subpopulations form based on location in the tumor as well as the genomic changes that occur. The second is that the cancer stages are not always clearly defined and at many times there is overlap between

the stages. Another consideration is that pathologists have different standards to separate the stages based on cancer type. For example, breast cancer tumors that measure between one and twenty millimeters would be considered Stage I, while tumors that measure between twenty and fifty millimeters would be determined as stage II and anything larger than fifty millimeters is diagnosed as Stage III. Lastly, size is not the only characteristic that defines the stage of a cancer. Doctors use results of several different diagnostic tests in order to properly utilize the TNM staging system. The three characteristics used are tumor size (T), if the cancer has spread to the lymph nodes (N), and lastly has the cancer has metastasized (M).

On a cellular and molecular level cancer stages are less defined. It is understood that cancer begins when basal cells spontaneously immortalize and no longer follow the Hayflick limit. Immortalization occurs most commonly with the loss of function of p53 and the activation of telomerases that via mutations in those genes [47]. These cells are not defined as cancerous but can merely divide indefinitely. In order to become cancerous, the cells must undergo transformation. The transformation stage of the cancer involves developing mutations that allow the cells to attain several hallmarks of cancer such as enhanced proliferation and invasiveness as well as becoming growth factor and anchorage independent. The final stage is metastasis. Metastasis occurs when angiogenesis promotes the growth of new blood vessels to allow the cancer cells that are no longer scaffolded to the extracellular matrix to acquire oxygen and nutrients. This allows for the more inner cells of the tumor overgrowth to survive in their environment. The tumor cells undergo a physiological and morphological change that occurs via a cytoskeletal reorganization through changes in microfilaments, intermediate filaments, and microtubules [48]. These cells can directly invade neighboring tissue, as well circulate into the bloodstream through the new blood vessels formed via angiogenesis to potentially colonize both neighboring and non-neighboring tissues and form satellite sites. Metastasis can occur from a single cell dissemination or a collective migration where several cells follow a leader cell [49]. Though there is some overlap with the clinically defined stages of cancer and the cellularly defined stages, the distinction is quite clear and based on the intention of

treatment versus understanding. While diagnosing cancer stage is based on size, reaching the lymph nodes and metastasis on the cellular level cancer stage is based on cellular events such as immortalization, transformation, and metastasis. Breast cancer is one of the most well-defined cancer progression models because it has distinct stages through which the cells progress [50].

## **Breast Cancer**

Breast cancer encompasses several cancers depending on the origin cell type. Breast cancer can be sub categorized into lobular, ductal, and less commonly Paget disease of the breast (nipple), phyllodes tumor (fat and connective tissue), and angiosarcoma (lining of the blood vessels). Breast cancer is the most found cancer in women in the United States in 2016 and had accounted for about 30 percent of all new cancers in women [7]. Breast cancer is also the most frequently diagnosed cancer in women in the world as of 2020 with more than 2 million new cases globally [8]. Though breast cancer is mostly found in women, it still accounts for the largest percent of new cancers globally in 2020 (11.7%) [9]. The current chance for women in the United States to develop breast cancer is about 12% [10]. The five-year survival rate of breast cancer in the US varies by stage. Breast cancer diagnosed at stage 0 and stage 1 has a 100% survival rate which then drops about 10% as the cancer progresses to stage 2. It then drops to a 72% five-year survival rate the cancer progresses to stage three and then the largest drop of 50% at stage 4 to a five-year survival rate of 22% [11].

There is also a disparity in breast cancer incidence, treatment, and outcome between ethnic groups within the United States.[74]. Incidences of breast cancer between Black women and non-Hispanic white women are about the same, with the rate of incidence is higher among Black women below the age of 45 while the rate of incidence is higher for non-Hispanic white women between the ages of 60 and 84.[74].

Asian/Pacific Islander women have the lowest incidence rate among all women [75,76]. There are several theories in the literature that explain the disparity in cancer incidences. Triple negative tumors which are

the most aggressive subtype of breast cancer have twice the rate of incidence in Black women, especially those in the younger category, compared to White women [74]. Other theories for this disparity include socioeconomic status as well as late stage of breast cancer at diagnosis [77]. These two theories are quite often linked. Because poverty is a key player in driving health disparity, it causes low-income women to be less likely to have a cancer screening and have a greater probability of a late-stage cancer diagnosis [78]. This link between poverty and higher mortality from breast cancer is observed in all ethnicities but is more prevalent in Black women due to larger proportion of Black women living in poverty compared to White women [79]. This could be due to several reasons such as living in disadvantaged areas without infrastructure such as primary care clinics as well as physicians [80]. Another reason is the potential lack of health insurance. Two studies found that Black women are twice as likely to be uninsured compared to White women [81,82]. Even if they are insured, the present health care providers may not be well enough equipped to provide treatment and adequate information to their patients [80,83-85].

When a patient is diagnosed with breast cancer, one of the first tests carried out is to determine if the cancer is Estrogen Receptor Positive (ER+). If the cancer found is determined to be ER+ then one treatment option is tamoxifen that acts as a competitor to estrogen and blocks the estrogen receptor in the breast cell. This same test is used to determine if the cancer is Progesterone Receptor Positive (PR+) and if either is present in the breast cancer tissue the cancer can also be labeled Hormone Receptor Positive (HR+). As mentioned previously, triple negative cancers are determined to be both ER- and PR- as well as HER2-. Human Epidermal Growth Factor Receptor 2 is a transcription factor that allows for the breast cancer to proliferate faster but is more susceptible to treatment. Unfortunately, breast cancer can become resistant to endocrine therapies as they progress which happens between 30-50% of patients partaking in these therapies [12]. This means that later stage cancers require more drastic treatments to cure the patient that include chemotherapy as well as half/full mastectomies. This is why early screening is so important. Unfortunately, the overarching issue is much larger in that it involves access to healthcare, infrastructure in underdeveloped areas, and addressing poverty on a wide scale. This is a larger undertaking that will

take a substantial amount of time and is why research in the field is crucial to addressing breast cancer in a three-pronged approach of addressing poverty and lack of infrastructure and research. The National Cancer Institute (NCI) has spent, on average, over 500 million dollars yearly on breast cancer research and with the addition of private funding the average reaches one billion dollars annually. Even with a billion dollars in research annually, how breast cancer becomes more aggressive and deadlier is still not fully characterized. What is known is that breast cancer undergoes a biological process known as Epithelial to Mesenchymal Transition (EMT) [14].

### **Epithelial to Mesenchymal transition.**

EMT is a reversible biological process, which allows for Epithelial cells to no longer interact with their basement membrane as well as convert into a mesenchymal phenotype. This mesenchymal phenotype allows for increased apoptotic resistance as well as increased migratory and invasive capacity [51]. There are three types of EMT noted as Type one, two and three. There are several similarities between the three types of EMT such as being reversible via a process known as Mesenchymal to Epithelial transition (MET), as well as the down regulation of epithelial markers and up regulation of mesenchymal markers. Each type of EMT has a clear distinction in biological function [14,52]. Type one EMT is involved in embryogenesis. More specifically these epithelial cells give rise to the primitive epithelium and followed by MET that give rise to secondary epithelia. It is speculated that the secondary epithelia can undergo differentiation via another round of EMT to form cells of connective tissue [14]. Type two is involved in the inflammatory response and wound healing. Unlike type one EMT, type two EMT is expressed over a longer duration and eventually reaches a crossroad. If the inflammation is attenuated, then type two EMT ceases but if the inflammation is not attenuated, this could lead to the destruction of the organ [14]. Finally, type three, which will be the focus in this thesis, is involved in cancer progression. As mentioned previously, the first step on a cellular level of cancer development immortalization occurs before type three EMT. Once immortalization occurs, the carcinoma is in situ until EMT allows for the transformation of the cells. Transformation gives rise to enhanced migratory and invasive capacities. The three types of

EMT differ in the signaling pathways involved as well as the major events that occur such as organ formation in type 1, tissue regeneration and organ fibrosis in type 2 and metastasis in type 3 [14, 52, 141].

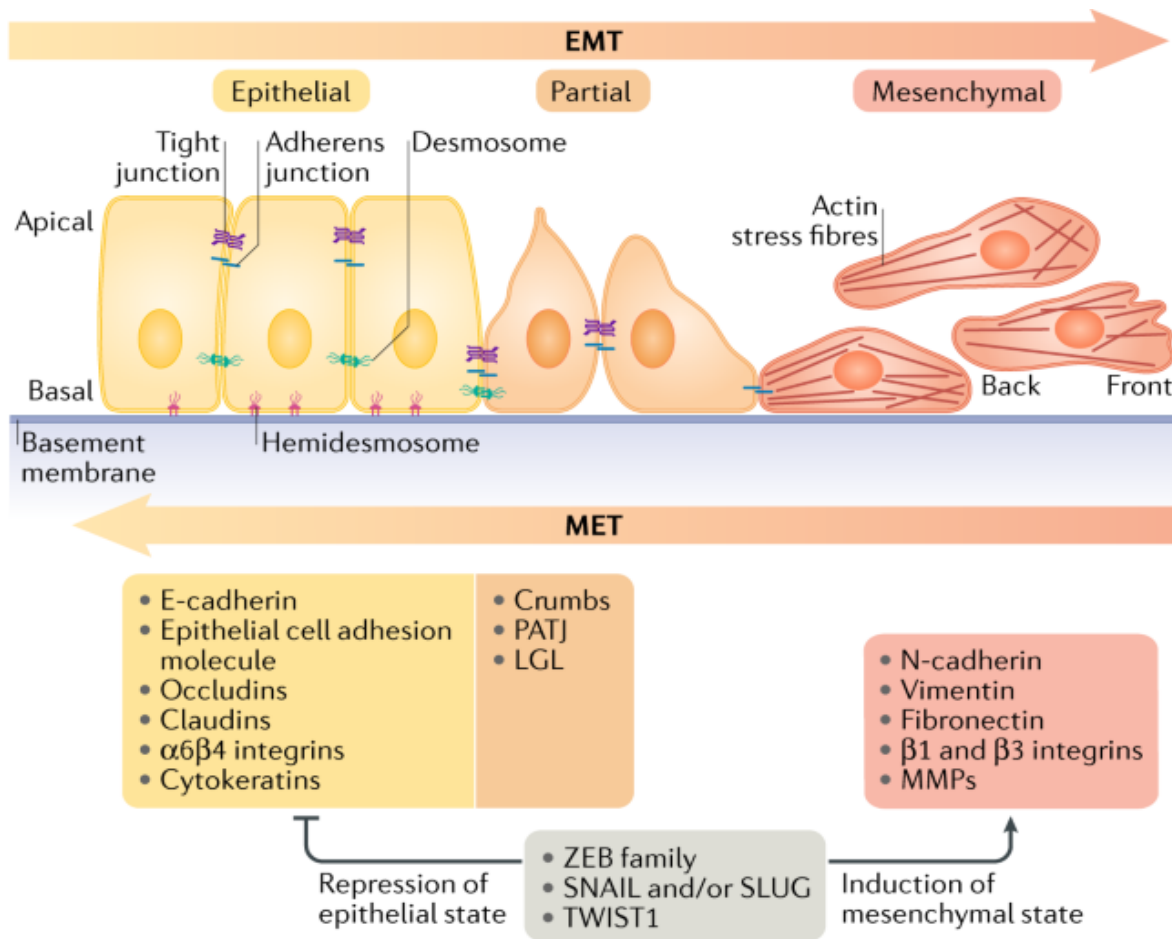
Breast cancer has been clearly shown to undergo the transdifferentiation of EMT in over 50 cell lines and follows a distinct trend [113]. It was observed that as breast cancer cells progressed there was an increased invasion capacity and metastatic potential, reduced or absent components of cell:cell adhesion complexes, as well as down regulation of Epithelial markers and upregulation of Mesenchymal markers [113].

EMT occurs as certain proteins and their respective mRNAs levels are altered within the cell, thus altering the phenotype of the affected cells. In the epithelial stage, proteins that are involved in cell-cell interaction and contact inhibition, such as  $\beta$ -Catenin and E-Cadherin, are at physiological levels and decrease as EMT progresses [15]. Contact inhibition allows for the cells to maintain an organized scaffold on the extra cellular matrix and inhibits overgrowth. As the cells progress towards a mesenchymal phenotype, genes involved in unchecked proliferation as well as migration and cell shape, such as Snail, Vimentin and N-Cadherin, are upregulated [15-16]. This can be seen in **Figure 1.3**. The genes mentioned previously as well as the genes in **Figure 1.3** are not the only genes involved with EMT, rather they are the genes that will be focused on in this thesis. This process results in cancer cells that are more migratory, proliferative, and invasive. This deadly trifecta gives little hope for treatment the farther the cancer progresses.

## **EMT genes**

The Epithelial phenotype related genes focused on in this thesis are  $\beta$ -Catenin and E-Cadherin.  $\beta$ -Catenin is a 92 kDa protein with two major functions. It was discovered in 1991 as a homolog of the armadillo protein in *Drosophila* that is involved in the mammalian cell adhesion complex with E-Cadherin [17]. It also has a role as a transcriptional co-regulator of the Wnt cascade which controls cell fate decisions [18,111]. E-cadherin is a 97 kDa protein and 120 kDa when matured. E-cadherin is calcium dependent





**Figure 1.3: EMT-MET Loop with Key Players.** Type 3 Epithelial to Mesenchymal Transition as well as Mesenchymal to Epithelial Transition is presented as a major biological process that begins with the presence of contact inhibition between cells via adherens and tight junctions via E-cadherin and  $\beta$ -Catenin proteins that are then downregulated while mesenchymal markers such as N-Cadherin, Vimentin, Fibronectin and MMPs are upregulated and allow for a progressive removal of contact inhibition as well as an attenuation of cell-matrix interactions and reorganization of cell organelle and cell structure to lead to a mesenchymal phenotype. This regulation is induced by the Zeb family of gene as well as SNAIL, Slug and TWIST1. The mesenchymal cells can then invade neighboring tissue as well as satellite sites via the blood stream where MET occurs by down regulating the mesenchymal markers and upregulation of epithelial markers such as via E-cadherin,  $\beta$ -Catenin Occludins, Claudins and Integrins. Figure taken from Dongre A and Weinber RA [127].

protein and is required for neighboring cells to maintain cell adhesion that eventually leads to form organized tissues [45]. This cell adhesion complex of  $\beta$ -Catenin and E-cadherin as well as the shorter isoform of p120 catenin allows for cells to maintain an Epithelial phenotype. As EMT progresses both

genes are downregulated decreasing the amount of cell adhesion. The mesenchymal phenotype related genes this thesis will focus on are *N-Cadherin*, *MMP2* and *Vimentin*.

N-Cadherin is 99.8 kDa protein and 130 kDa when matured, that is also calcium dependent protein that interacts with the longer isoform of p120. During EMT a “cadherin switch” occurs where E-Cadherin is downregulated, and N-Cadherin is upregulated allowing for the cancer cells to become more metastatic and invasive [45-46]. This change occurs via interconnectivity of the cells via N-Cadherin that allows for the cells to have collective cell polarity and coordinated cytoskeletal activity. This is how a leader-follower relationship between the cells occurs [143].

*MMP2*, also known as Matrix Metalloproteinase 2 is a 74 kDa protein that as the name suggests is a proteinase that degrades the major structural and most abundant component of basement membranes type IV collagen. This degradation allows for cancer cells to no longer be scaffolded to the ECM and eventually leads to tumor formation, invasion, and metastasis.

Vimentin is a 57 kDa protein that plays a role in maintaining cell shape by being involved in integrity of the cytoplasm and stabilizing cytoskeletal interactions. This includes structural support, attachment as well as migration [56]. In EMT, Vimentin concentration is very low in the epithelial stage and increases in the mesenchymal stage [16]. Due to this increase in concentration, Vimentin is considered a canonical biomarker of type 3 EMT [16,57,58]. Upregulation of Vimentin levels has also been shown to be associated with poor prognosis in AML [57].

These are not the only EMT gene related genes currently known. Other genes such as Fibronectin, Occludins and Claudins are all also determined to be EMT genes [53]. Some groups have determined that there are 82 EMT related genes [53] while others have found 130 related EMT genes [54]. A more extensive study of over 7200 genes utilized the top 200 EMT related genes based on a 1.2 absolute fold change from 12 cell lines across six cancer types [55]. Interestingly, all the EMT genes mentioned previously have been shown to be regulated by a single gene known as snail family transcriptional

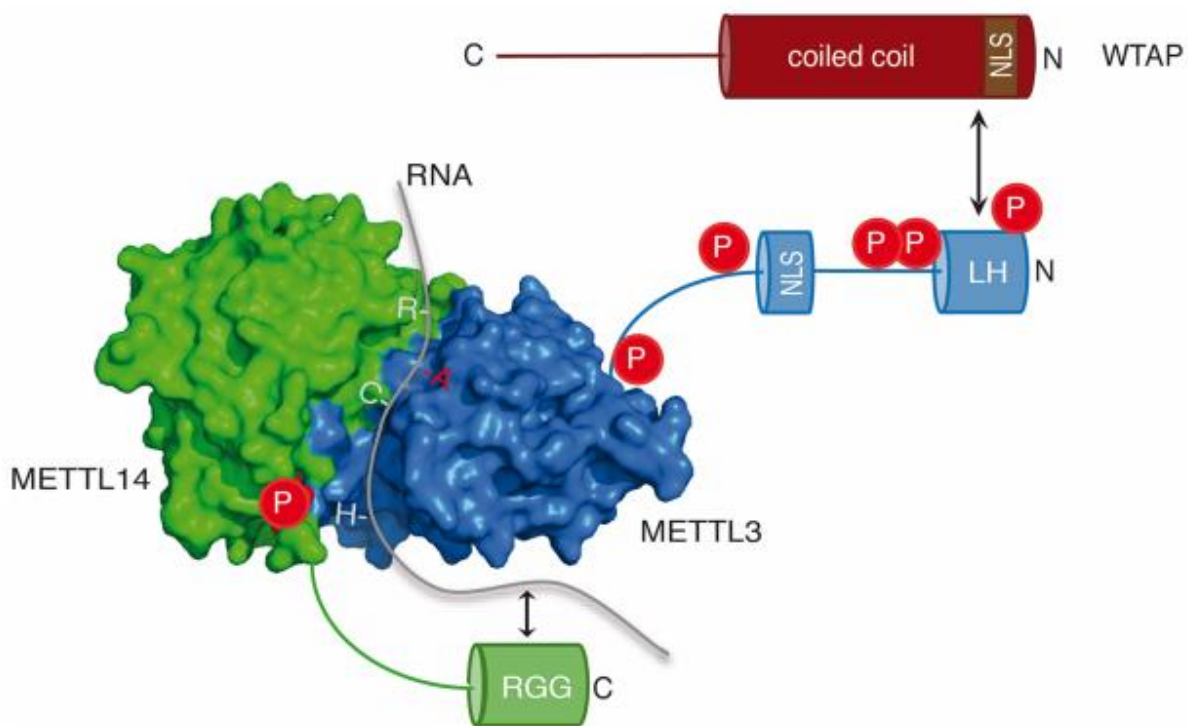
repressor 1 (*SNAIL*) [93,112]. *SNAIL* has been shown to down regulate epithelial marker *E-Cadherin* and upregulate mesenchymal markers *Vimentin*, *MMP2/9*, and *Fibronectin* [97,103,104]. It is clear that *Snail* plays a central role in regulation of major EMT genes and as more studies are done, both EMT and MET will continue to emerge as more complex biological processes than previously thought with more players being involved than currently found. As more EMT related genes are being determined, the next level of characterization is to understand different modes of regulation of these genes during EMT. One notable mode of regulation of EMT genes that is currently emerging is the RNA modification m6A [24].

## **m6A**

m6A is a reversible RNA modification in which the nitrogen on position 6 of adenosine is methylated. The m6A modification was discovered to be the most found mRNA modification nearly 50 years ago [4]. This modification has been shown to affect translation efficiency, alternative splicing, mRNA stability, nuclear export as well as P-body storage [19]. This modification occurs via m6A methylases known as m6A writers. Two major m6A writers are Methyltransferase-3 ( *METTL3*) and Methyltransferase-16 ( *METTL16*) with *METTL3* having a larger number of targets for methylation. *METTL16* has been shown to methylate adenosines in mRNA, rRNA as well as snRNA while *METTL3* has been shown to methylate adenosines in mRNA, circRNA, miRNA as well as lncRNA [59,60]. *METTL3* is part of the semi-characterized complex that contains Methyltransferase-14 ( *METTL14*) as well as Wilms tumor associated protein (*WTAP*). These 3 proteins are the core of the complex with each playing major and diverse roles in methylating mRNAs. *METTL3* is responsible for the catalytic activity via its Rossmann-like fold domain that is found in other class I methyltransferases and with its substrate S-adenosylmethionine (SAM) it can methylate the target pre-mRNA. *METTL3* also contains a nuclear localization signal allowing it to enter the nucleus. *METTL14* does not have a nuclear localization signal and is chaperoned by *METTL3* in order to enter the nucleus. *METTL14* is utilized for substrate binding (mRNA scaffolding) to increase

efficiency of the complex. WTAP has its own nuclear localization signal and plays a role in bringing the complex to the target pre-mRNA [20]. **Figure 1.4** models the METTL3- METTL14-WTAP complex with an RNA substrate.

The consensus sequence for the m6A modification via the METTL3- METTL14-WTAP complex is DRACH where D represents an Adenosine, Guanine or Uracil, R represents an Adenosine or Guanine, and H represents an Adenosine, Cytosine or Uracil respectively [21]. This is a commonly found sequence and only about 10% of the center Adenosine in DRACH is methylated [21]. Though there are many other m6A writers such as METTL5 and TrmM that methylate rRNAs and tRNAs respectively, METTL3 is the most widely studied m6A writer [60,61].

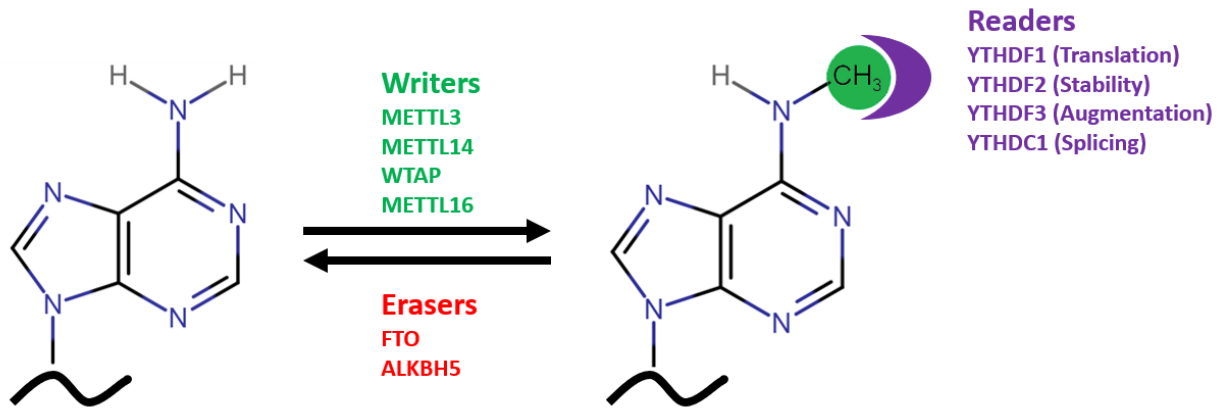


**Figure 1.4: The METTL3- METTL14-WTAP Complex.** The METTL3- METTL14-WTAP complex is the most studied m6A writer complex. METTL3 is responsible for catalytic activity at the m6A labelled with a red A, as well as chaperoning METTL14 into the nucleus with its nuclear localization signal. METTL14 is responsible for scaffolding the RNA target for m6A to increase transcription efficiency via its arginine-glycine-glycine motif. WTAP has its own nuclear localization sequence but is not fully characterized. Other proteins in the complex have been observed such as KIAA1429, VIRMA, USP5 but are not yet characterized. Image taken from Schöller E., et al [20].

The m6A modification is reversible by the m6A erasers fat mass and obesity related portion (*FTO*) and alkylation repair homolog 5 (*ALKBH5*) also known as demethylases. Both m6A erasers have been involved in demethylating mRNAs in nuclear speckles [62,63]. Both *FTO* and *ALKBH5* have been found to have distinct pathways and intermediates when demethylating their targets [117]. Both *FTO* and *ALKBH5* use Iron as well as 2-Oxoglutarate and O<sub>2</sub> to convert the methyl group in m6A into a hydroxymethyl group that eventually separates into a formaldehyde and a demethylated adenosine at position six [134]. The difference occurs when the hydroxymethyl group is formed known as N6-hydroxymethyladenosine (hm6A). *ALKBH5* catalysis only results in an unmethylated adenosine while *FTO* produces a mixture of N6-formyladenosine (f6A) and an unmethylated adenosine [135-137]. Though both erasers have been shown to only target m6A modifications, currently no consensus sequence has been found for either m6A eraser [134,136,137]. Discrimination between targets of each m6A eraser has been observed [118]. This group determined that an m6A transcript alone has negligible demethylase activity with both *FTO* and *ALKBH5* preferring a sequence of GRACH with *FTO* having consistently higher demethylase activity [118]. The discrimination of *ALKBH5* and *FTO* is further observed in the location of the m6A as well. A different group determined that *FTO* targets the first encoded nucleotide adjacent to the 7-methylguanosine cap known as N6,2'-O-dimethyladenosine (m6Am) while *ALKBH5* does not [138]. This selectivity of sequence still does not address why *FTO* has been shown to target genes linked to obesity while *ALKBH5* has been shown to target genes linked to spermatogenesis, and both have been associated with several cancer types and their prognosis [27, 118]. There is still much to be studied in both the characterization of the m6A erasers as well as the mechanism of discrimination whether that be due to a consensus sequence, gene target or another mode recognition.

The m6A readers interact with m6A modification to induce a regulatory change. The most well characterized m6A readers are the YTH family of proteins. These proteins do not induce a regulatory change independently but rather recruit the relative machinery as well as translocate the mRNA transcript in order to affect their respective biological processes. YTHDF1 enhances translation of mRNA

transcripts with an m6A by promoting ribosome assembly and interacting with the initiation factor EIF3 [22,119]. Interestingly, YTHDF1 is observed to have a consensus sequence for its m6A target. It has been observed that YTHDF1 selectively recognizes and binds to the GRAC site, as mentioned before the R represents a G or A [119]. YTHDF2 regulates the stability of mRNA transcripts with m6A through its carboxyl-terminal domain that binds to m6A, while its amino terminal domain that is responsible for transporting the mRNA to the decay site and switch the mRNA from the translation state to the degradation state [120]. YTHDF2 also has a consensus sequence that differs from YTHDF1 that is RRACH where R represents an A or G, and H represents an A, C or U [120]. YTHD3 has been observed to augment both YTHDF1 and YTHDF2 activity by interacting with each reader or independently by interacting with EIF4A3 for enhancing translation efficiency or interacting with the GAC m6A site and translocating the m6A mRNA transcript to RNA decay sites [121]. YTHDF3 has been shown to also share targets with YTHDF1 and YTHDF2 [121]. YTHDC1 recruits splicing factor Serine and Arginine rich splicing factor 3 (*SRSF3*) while blocking Serine and Arginine rich splicing factor 10 (*SRSF10*) to nuclear spots allowing for a distinct splice form to be released from the nucleus into the cytoplasm [122,123]. YTHDC1 also recognizes the GAC sequence of m6A but a consensus sequence for YTHDC1 has not been observed [123]. It has also been observed that m6A is involved in nuclear export as well as P-body storage of mRNA messages via readers HNRNPA2B1 and YGF2BP1-3 respectively but these readers have not yet been fully characterized [23]. A diagram of m6A writers, erasers, and readers can be seen in **Figure 1.5**.



**Figure 1.5: m6A Writers, Erasers and Readers.** m6A is a reversible RNA modification that occurs on position 6 of adenosine where the Nitrogen is methylated via m6A writers and demethylated by m6A erasers. The m6A modification is recognized by m6A readers that will then recruit their respective machinery as well as translocate the RNA to the site of regulation to induce a regulatory change on the RNA. Image modified from Gokhale, N. & Horner, S [141].

m6A via the METTL3- METTL14-WTAP complex has been shown to be involved in several physiological processes such as embryo and organ development, metabolism, immune regulation, T-cell homeostasis as well as cancer [86-89]. This finely tuned mechanism has large implications across multiple major biological processes. By methylating certain messages involved in these processes, a crucial transcript can have its translation enhanced via m6A, or the message of an inhibitor can be flagged for degradation to allow for a biological process to proceed unhindered. When attempting to characterize m6A in different biological processes, the most common focus is the m6A writer METTL3. METTL3 as mentioned before is the protein responsible for catalytic activity. Though there are several other players in the Mett3- METTL14-WTAP complex, METTL3 is manipulated in order to affect m6A levels in most studies. This includes being knocked down via CRISPR/Cas9 as well as shRNA and being overexpressed ectopically via plasmid transfection. METTL3 has also been shown to enhance translation of oncogenic transcripts such as Epidermal Growth Factor Receptor, *EGFR*, and PDZ-binding motif known as TAZ

independently of its methyl transferase activity via recruitment of eukaryotic translation initiation factor 3 (eIF3) to the translation initiation complex [87, 114].

## m6A and Cancer

Several studies have begun to link m6A modifications and the progression of cancers through EMT [24-26]. This still does not address why there are many conflicted results in regard to m6A promoting or suppressing different cancers. Currently in the field, there is data to suggest m6A promotes tumor progression in breast [21,27-31], pancreatic [32], gastric [33-35], lung [36-39] and Acute Myeloid Leukemia (AML) [25,27,41] cancer, as well as data that suggests m6A inhibits tumor progression in breast [29,40,22-24], pancreatic [27,25], lung[21] and cervical [27], and other[26,42]. cancers. Though m6A has been shown to both promote and suppress different cancer types, it has also been shown to promote and suppress cancer in the breast, lung, and pancreas. The controversy in the field about whether m6A suppresses or promotes cancer is illustrated in **Figure 1.6**.

m6A promotes cancer			m6A suppresses cancer		
Cancer Type	Proteins involved	m6A's affect on Phenotype	Cancer Type	Proteins involved	m6A's affect on Phenotype
Ovarian	METTL3	Increase in tumor invasion/growth	Endometrial	METTL3	Decrease in proliferation/migration
Bladder	METTL3	Increase in tumor growth	Nasopharyngeal carcinoma	BH	Increase in apoptosis/cell cycle arrest
csCC	METTL3	Increase in cell proliferation	Cervical	FTO	Decrease in cancer development
Leukemia	METTL3/14	AML development	Glioblastoma	ALKBH5	Decrease in tumorigenicity
<b>Breast</b>	<b>METTL3</b>	<b>Increase cancer progression</b>	<b>Breast</b>	<b>FTO/ALKBH5</b>	<b>Decrease in tumor growth</b>
<b>Lung</b>	<b>METTL3</b>	<b>Increase in tumor invasion</b>	<b>Lung</b>	<b>FTO</b>	<b>Decrease in proliferation/invasion</b>
Colorectal	METTL3	Increase in tumorigenicity	Liver	METT14/YTHDF2	Decrease in tumorigenicity
<b>Pancreatic</b>	<b>METTL3</b>	<b>Increase in cancer development</b>	<b>Pancreatic</b>	<b>ALKBH5</b>	<b>Decrease in tumor proliferation</b>
ccRCC	FTO	Increase in cell proliferation	Cervical	FTO	Decrease in chemoradiotherapy resistance

**Figure 1.6: m6A and its Currently Understood Role in Cancer.** This table presents the challenge of navigating the literature of m6A and its role in cancer. The left side presents phenotype changes found for m6A's promotion of cancer via writers and in negation of m6a eraser activity. The right presents phenotype found for m6A's repression of cancer via m6A writers, erasers, and readers. This image was developed from Yu, T, et al [132].



There are many layers to understanding this controversy. The first is to define what is meant by promoting or suppressing cancer. Promoting and suppressing cancer are very overarching statements that can cover many different phenotypes and biological processes. As shown in **Figure 1.6**, the end result of the studies shown are a change in invasion, migration, proliferation, tumor growth, cancer development and tumorigenicity. That still does not include apoptosis resistance, avoiding immune response, induction of angiogenesis as well as other hallmarks of cancer. There is also literature that suggests that some of these characteristics of cancer form a dichotomy such as proliferation and migration [64]. Another layer that adds to the convulsion of m6A and its role in cancer is the methods of manipulation in order to bring about a change in m6A for further study.

The most common target of study is METTL3 due to its catalytic activity as well as its role as a chaperone for METTL14 to enter the nucleus via its nuclear localization signal. METTL3 has been shown to methylate *AXL*, a tyrosine-protein kinase receptor that allows for cancer to escape immune response as well as attain drug resistance, allowing for enhanced translation that leads to an overall increase in tumor growth as well as cancer invasion [65,66]. Decreasing m6A levels through knockdown of METTL3 via shRNA in human endometrial adenocarcinoma cells led to increased phosphorylation of AKT, FOXO1 and p27 [67]. *AKT*, also known as protein kinase B, is a kinase that is involved in cell growth and proliferation through *mTORC1* as well as VEGF secretion and angiogenesis [68]. *FOXO1* is a transcription factor that regulates cellular metabolism and is inhibited by phosphorylation by AKT. *p27* is a transcriptional regulator that regulates cell cycle by binding to and inhibiting Cyclin-CDK and also regulates apoptosis by arresting the cell cycle at G1, [69,70]. Increased phosphorylation of AKT, FOXO1 and p27 lead to an increase in cell proliferation, as well as tumorigenicity [67]. METTL3 being the crux of the METTL3- METTL14-WTAP complex as well as being involved in many hallmarks of cancer makes it a great target to attenuate m6A levels and determine m6A's diverse role in cancer progression.

Other studies target m6A erasers such as ALKBH5 and FTO. In one study, it was observed that increased m6A levels caused by less FTO expression inhibits PGC-1 $\alpha$ , Peroxisome Proliferator-activated receptor-gamma coactivator, by reducing its mRNA stability leading to an overall increase in cell proliferation [71]. *PGC-1 $\alpha$*  is a transcription coactivator that plays a major role in regulation of cellular energy metabolism [72]. In a different study it was observed in lung squamous cell carcinoma (LUSC), that increased FTO leading to a decrease in m6A caused increased mRNA stabilization of Myeloid Zing Finger Protein 1 (*MZF1*) [73]. Furthermore, some studies determine m6A's role in cancer progression by modifying upstream regulators of m6A such *c-MYC* and SPI1. Many of these genes that are affected by demethylation have secondary and even tertiary roles within the cell that could potentially lead to conflicting results.

Not only are the writers and erasers involved in both the promotion and suppression of cancer but the readers as well. In one group, YTHDF2 has been found to promote Liver cancer stem cell phenotype in Hep3B and Huh7 cells [128]. This group knocked down YTHDF2 via shRNA and observed that both the tumor spheres per 1000 cells decreased as well as the CD133+ cells detected via flow cytometry.[128]. Another group found that in the gastric cancer AGS cell line, YTHDF2 decreased stability of the transcription factor Forkhead box protein C2, *FOXC2*, which lead to an attenuation of proliferation and migration [129]. They observed an opposite effect in the human gastric carcinoma cell line MGC-803 [129].

Another consideration is cancer type. Cancer types can be separated firstly by liquid cancers that are constant suspension or solid cancers that are adherent. Another mode of separation in cancer types is the organ itself as not all cancers within the solid or liquid description follow identical pathways or mutation. This is especially true in regards to m6A. For example, in liquid cancers such as acute myeloid leukemia and lymphoma m6A has been shown to increase proliferation in both cancer types and increased apoptotic resistance in leukemia and increased drug resistance in lymphoma [130-131]. In multiple myeloma, MM, m6A was shown to suppress tumorigenicity and stem cell phenotype [132]. Furthermore, m6A has been shown to both promote and suppress cancer in breast, lung, and pancreas [9,21,25,28,29,32,36,39,133].

Lastly, subpopulations of cells with unique genomic characteristics can occur within a cancer type based on environmental factors as well as the nature of mutations developed. The data presented in this thesis has shown that cell type within the same cancer type as well as the same parental line of origin could also have differing effects of m6A and the progression of cancer. This may be due to stage of the cancer and no similar study has been presented in the literature.

### **m6A and EMT targets**

Over the past several years, the field has been able to demonstrate a direct link between m6A and EMT. As mentioned before, EMT is a reversible major biological process in which cells transition from an epithelial phenotype to a mesenchymal phenotype. This occurs when epithelial markers such as E-cadherin are downregulated and mesenchymal markers such as N-Cadherin and Vimentin are upregulated. In breast cancer, the overexpression of m6A writer KIAA1429 and eventual augmentation of m6A levels lead to an increase protein expression of N-Cadherin and a decrease in protein expression of E-Cadherin therefore promoting EMT [140]. Similarly, it has also been observed in gastric cancer that with the knockdown of METTL3 and eventual attenuation of m6A, E-cadherin increased protein expression while N-Cadherin and Vimentin a decrease in protein expression [33]. Another group saw the same results in lung cancer and when treating their cells with cigarette smoke extract observed an increase in METTL3 and an eventual augment of m6A that negatively affected E-cadherin protein expression while positively affecting protein expression of N-Cadherin and Vimentin [34]. These three groups presented evidence that m6A promotes EMT. Unfortunately, just as cancer progression and m6A's relationship requires more study due to conflicting evidence, the link between EMT and m6A is no different. In two different thyroid cancer cell lines, the knockdown of METTL3 and eventual attenuation of m6A lead to the opposite effect observed in gastric and lung cancer [139]. With the attenuation of m6A levels E-cadherin protein expression increased while protein expression levels of N-Cadherin and Vimentin decreased [139]. The link between m6A and EMT has eluded researchers not only when looking at EMT targets but also

regulators of those targets as well. One of the regulators of EMT that is linked with m6A is transforming growth factor beta (TGF- $\beta$ ) [24]. TGF- $\beta$  is a cytokine involved in regulating inflammation as well as stem cell and T-cell differentiation [90-92]. When HeLa and HepG2 cells were treated with TGF- $\beta$  for three days, the cells were determined to be undergoing EMT [24]. Those same cells undergoing EMT were observed to have a 20% increase in m6A levels in mRNA in the HeLa cells and a 14.9% increase in HepG2 cells thus showing that m6A increased as EMT occurs [24]. They had also observed a decrease in protein expression of epithelial marker E-cadherin and increase in mesenchymal marker Fibronectin with the introduction of TGF- $\beta$  but when they knocked down METTL3 with CRISPR/Cas9 in those same HeLa cells, the introduction of TGF- $\beta$  did not show a significant change in protein expression of either marker compared to the control [24]. They had also observed that with the knockdown of METTL3 and TGF- $\beta$  treatment that the HeLa cells showed a decreased capacity for migration and invasion compared to wild-type HeLa cells with TGF- $\beta$  treatment [24]. This demonstrates that TGF- $\beta$  alone was not enough for the cells to progress through EMT and that METTL3 and its ability to methylate mRNA messages with m6A was necessary for EMT. From there, the group compared 84 related EMT genes and 61 genes that showed a 2.0-fold m6A change during EMT and found four genes that overlapped between the two groups. The most striking SNAIL commonly referred to as Snail. Snail was shown to have an m6A in coding region that decreased with the knockdown of METTL3 [24]. This m6A site was further determined to interact with m6A reader YTHDF1 that is responsible for translation efficiency. These results were further confirmed with polysome profiling that indeed m6A on Snail in the coding region led to higher translation efficiency [24]. Snail plays a central role in EMT by repressing Epithelial markers such as *E-cadherin*, *Occludin*, *Claudins* and *Mucin1* as well Tumor suppressors *RKIP* and *PTEN* [93-102]. *Occludin* and *Claudins* are integral membrane proteins that play a major role in forming cellular tight junctions that appear where two cells meet. *Mucin1* is membrane-associated, surface protein that plays a role in lubricating and stabilizing the mucus on the cell surface which assists in regulating cell adhesion [107]. *RKIP*, RAF-kinase inhibitor protein, is as its name suggests, modulates cellular growth, apoptosis, cell motility, genomic integrity and drug resistance via inhibiting several pathways such as the

*MAPK* (Raf-1/MEK/ERK) and the *NF-κB* (Nuclear factor kappa B) signaling pathways [108-110]. PTEN, phosphatase and tensin homolog, is a tyrosine phosphatase that negatively regulates the *AKT/PKB* signaling pathway. Snail also has been shown to upregulate Mesenchymal markers *Vimentin*, *Fibronectin*, *MMP2*, *MMP9* and EMT inducers zing finger E-box binding homeobox 1, *ZEB-1*, and lymphoid enhancer binding factor 1, *LEF-1* [101-106]. *ZEB-1* as the name suggests utilizes its zinc fingers to bind to DNA specifically at the E box in the promoter of E-cadherin and then recruits co-repressors to block the transcription of E-cadherin [115]. *LEF-1* binds to T-cell receptor alpha locus, *TRCA*, and enhances its activity. In EMT it has been implicated in expression of microRNAs involved with EMT [116]. By m6A enhancing translation of the major EMT regulator Snail, m6A has been shown to promote EMT through multiple EMT markers and pathways. Unfortunately, there is also evidence that suggests that m6A suppresses EMT in thyroid cancer. This controversy in the field leaves much to be studied in order to fully characterize m6A and its relationship to EMT.

## Conclusion

As more groups characterize breast cancer progression via EMT as well as the role of m6A in this transdifferentiation, the field should begin to see a clearer picture of the relationship between the three subject matters. Unfortunately, that is not the case due to contradictions in the overall results in the field in regards to m6A's role in cancer progression. As shown in **Figure 1.6**, m6A has been shown to both promote and suppress cancer progression depending on cell type. It was observed to promote cancer in the ovary, bladder and colon and suppress cancer in cervix, liver, and brain. It was also observed to both promote and suppress cancer breast, lung, and pancreas. Researchers have been unable to fill the gap in knowledge for several years now and as more data is observed and published, more confusion arises. This occurs due to the amount of data in the field as well as results that show conflicting results in regards to m6A and its relationship with cancer progression. Currently the field has been presented with a monumental task of both consolidating and extrapolating data from this plethora of global research in

order to reach a widely accepted conclusion of the cause of dynamic and often conflicting role of m6A in cancer progression. This thesis aims to begin a paradigm shift in the understanding of m6A and its dynamic role in cancer progression so that future research can continue while being equipped with the correct characterization so that potential treatments in not just cancer but all m6A related conditions can be developed efficiently and effectively.

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## **Chapter 2: m6A and its Relationship with Breast Cancer Progression**

## Introduction

The National Cancer Institute (NCI) has spent, on average, over 500 million dollars yearly on breast cancer research and with the addition of private funding the average balloons to one billion dollars annually. Despite this, there is still little understanding on both how and why breast cancer becomes more aggressive and deadlier at an alarming rate. The five-year survival rate drops 20 percent between stage 2 and 3 and an additional 50 percent between stage 3 and 4 [1]. The mode of this deadly progression typically involves a process known as Epithelial to Mesenchymal Transition (EMT) [32]. As mentioned before, EMT occurs as certain proteins and their respective mRNAs are altered within the cell, thus altering the phenotype of the affected cells. During the epithelial stage, cells exhibit cell-cell adhesion and contact inhibition due to basal levels of  $\beta$ -Catenin and E-Cadherin. This ensures the cells are scaffolded onto the ECM and blocks overgrowth. As EMT progresses  $\beta$ -Catenin and E-Cadherin are decreased [2]. As the cells progress towards a mesenchymal phenotype, genes involved in unchecked proliferation as well as migration and cell shape, such as Snail, Vimentin and N-Cadherin, are upregulated [2, 3]. Ultimately, this process results in cancer cells that are more migratory, proliferative, and invasive, a deadly trifecta that has little hope of treatment the further the cancer progresses.

The N6-methyladenosine (m6A) modification was discovered to be the most common mRNA modification nearly 50 years ago [4]. This modification has been shown to affect translation efficiency, alternative splicing, mRNA stability, nuclear export, as well as P-body storage [5]. This modification occurs via methyltransferases known as m6A writers such as METTL3 and METTL16 with METTL3 having a larger number of known targets for methylation. METTL3 is part of a semi-characterized complex that contains METTL14 as well as Wilms tumor associated protein (WTAP). These 3 proteins are the core of the complex with each playing major and diverse roles in methylating mRNAs. METTL3 is responsible for the catalytic activity via its Rossmann-like fold domain that is found in other class I methyltransferases and with its substrate S-adenosylmethionine (SAM) it can methylate the target pre-

mRNA. METTL3 also contains a nuclear localization signal allowing it to enter the nucleus. METTL14 does not have a nuclear localization signal and is chaperoned by METTL3 to the nucleus. METTL14 is utilized for substrate binding (mRNA scaffolding) to increase efficiency of the complex. WTAP has its own nuclear localization signal and plays a role in bringing the complex to the target pre-mRNA [34]. The consensus sequence for the m6A modification via the METTL3- METTL14-WTAP complex is DRACH where D is an Adenosine, Guanine or Uracil, R is an Adenosine or Guanin and H is an Adenosine, Cytosine or Uracil respectively. This is a commonly found sequence and only about 10% of the center Adenosines in DRACH are methylated [6]. The m6A modification is reversible by the demethylases known as m6A erasers fat mass and obesity related portion (FTO) and alkylation repair homolog 5 (*ALKBH5*). The m6A readers such as the YTH family of proteins interact with the m6a to induce a regulatory change. Rather than directly being responsible for the regulatory change, m6A readers recruit their respective regulatory machinery as well as translocate the RNA to the site of regulation. YTHDC1 is used in splicing as mentioned before by recruiting SRSF3 and blocking SRSF10. YTHDF1 is for translation by recruiting the relevant machinery while YTHDF2 is used for RNA stability by blocking degradative processes [7]. M6A has also been shown to be involved in nuclear export and storage of mRNA messages [5].

As mentioned before, several studies have begun to link m6A modifications and the progression of cancers through EMT [8,23,25]. However, there are many conflicting results with regard to the m6A modification promoting or suppressing different cancers. As stated before M6A has been shown to promote tumor progression in breast [6,10-13, 31]. pancreatic [14], gastric [15-17], lung [18-21] and Acute Myeloid Leukemia (AML) [10,22,23] cancer. It has also been observed that M6A inhibits tumor progression in breast [10, 12, 22-24], pancreatic [10,23], lung [21] and cervical [10] and other [25,26] cancers. This dichotomy leads us to believe that there may be additional factors regulating m6A's specific role in cancer. Our study shows that the stage of the cancer may be one of the reasons behind these conflicting results. As m6A's role in cancer is being further characterized, these results, as well as future



studies must be consolidated to better understand the targets of the modification as well as the mechanisms affecting cancer progression.

This study aimed to directly link m6A to EMT in breast cancer progression and characterize the unique and diverse roles m6A has at differing stages of that progression. In order to do this, we elected to use the MCF10 model of breast cancer progression in our study. The MCF10 model was developed by the Karmanos Cancer Center at Wayne State University in 1984. The MCF10 Model is derived from benign proliferative breast tissue that spontaneously immortalized in a 36-year-old Caucasian female and is widely used to study cancer progression as well as EMT [27,28]. We chose to study three cell lines from the MCF10 cell model, each representing different stages of progression (**Figure 2.1.A**). The MCF10As, which are spontaneously immortalized MCF10 cells, are early-stage cancer cells that have a basal-like cell structure. They do not express estrogen receptors and are not tumorigenic [27]. These are very early-stage cancer cells that are our starting point in understanding the regulation required for progression. The next cell line in progression is MCF10AT1's which are slightly more proliferative as well as migratory than the MCF10A's. This is due to the ectopic expression of a mutated HRas (G12V) via stable integration into the MCF10A cell line. Last are the MCF10Ca1h's, which are late-stage tumorigenic cells that are extremely aggressive, proliferative, and capable of metastasis. They were developed from the MCF10AT1 cells via serial mouse xenografts that led to high genomic instability as well as several copy number gain and loss mutations. Thus, these three cell lines represent three distinct stages of breast cancer progression which have all arisen from the same parental cell line.

Utilizing these three cell lines from the established MCF10 model, we knocked down METTL3 via CRISPR/Cas9. The resulting cell lines were then used to determine changes in proliferation and migration as well gene expression in response to decreased m6A levels. This study is the first to show several different effects that m6A has on distinct stages of a well-defined breast cancer progression model. These effects are not only unique but are opposite depending on stage. We found that m6A plays a unique and

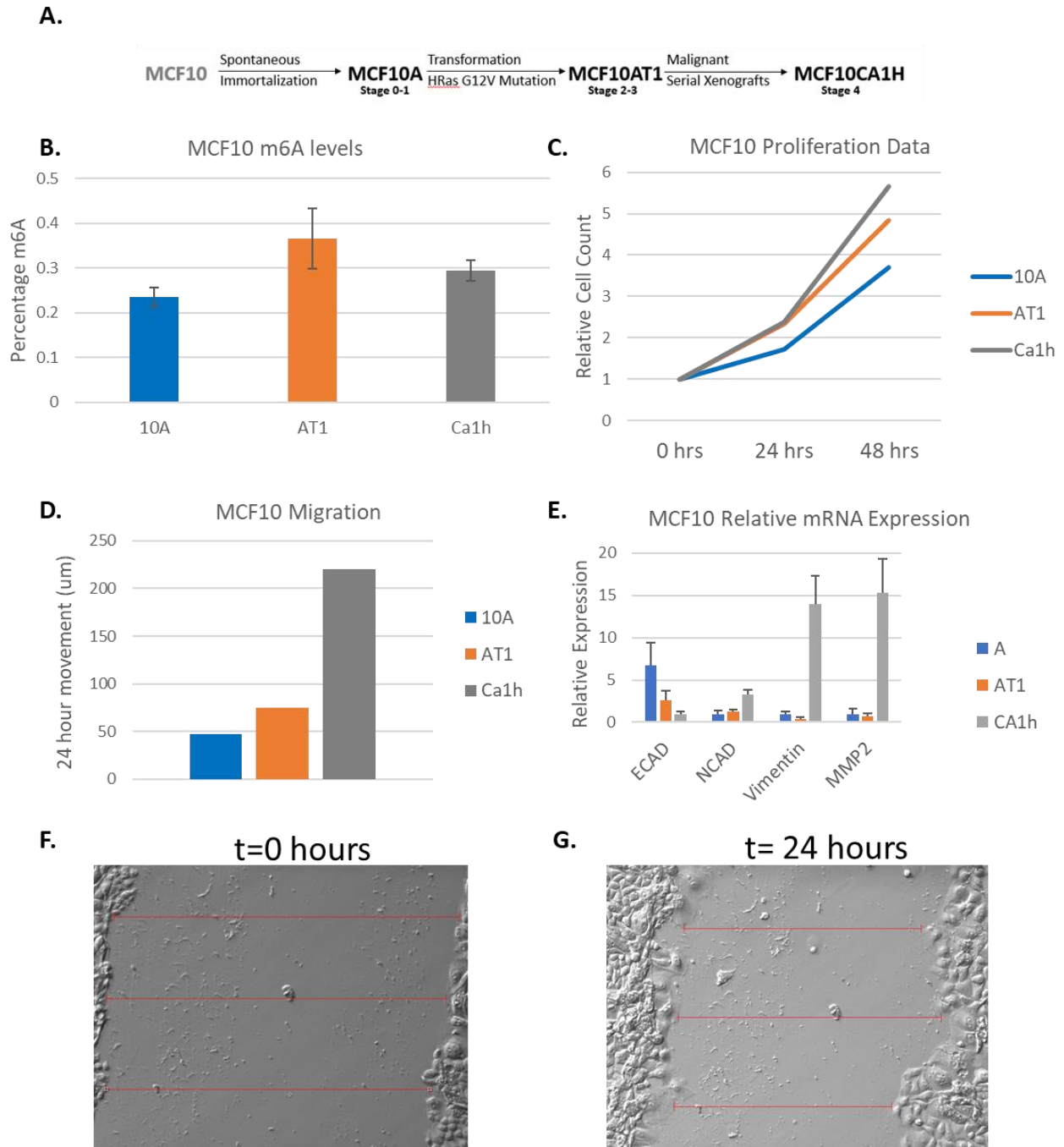
dynamic role in breast cancer progression. With this characterization we can both consolidate, as well as further understand, the discrepancy in the field of m6A and its effect on cancer progression.

## Results

### *Characterization of the MCF10 Breast Cancer Model*

We first wanted to determine the m6A levels of each of our cell lines as a baseline for the study. PolyA RNA was extracted from the MCF10A, MCF10AT1 and MCF10Ca1H cell lines, rRNA depleted, and m6A levels determined via LC-MS [13]. As shown in **Figure 2.1..2**, the MCF10AT1 lines had the highest percentage of its adenosines methylated with m6A followed by the MCF10Ca1h and the MCF10A. This initial increase of m6A levels from MCF10A to MCF10AT1 followed by a decrease in m6A levels between the MCF10AT1 and MCF10Ca1h suggests that m6A may have a dynamic role in the progression of this breast cancer model.

Next, we characterized our cell lines to verify that our model of breast cancer progression showed increasing proliferation and migration as we expected [29]. Proliferation was tested by counting the cells every 24 hours over a 72-hour time course while a wound healing assay was used to determine the extent of migration of the three cell lines. As shown in **Figure 2.1**, we observed an increase in proliferation (**Figure 2.1.C**) and migration (**Figure 2.1.D**) as the cell lines progressed to more tumorigenic with the MCF10A cell line showing the least and MCF10Ca1h showing the greatest. This is to be expected as it is known cancer becomes more proliferative and migratory as it progresses and gives credence that we have a well-chosen working model that follows the characterization of breast cancer on the phenotypical level.



**Figure 2.1 The MCF10 Breast Cancer Model:** **A.** The MCF10 Breast cancer model used for this study starting from the original cells to our late-stage cancer cells. **B.** LC-MS/MS of mRNA nucleosides isolated from to show percent m6A levels of our parental lines in our MCF10 Breast Cancer Model. **C.** Preliminary Proliferation Cell count data on our parental cell lines in order to confirm our breast cancer model follows established trends that occur with progression. **D.** Preliminary Migration/Scratch Assay data on our parental cell lines in order to confirm our breast cancer model follows established trends that occur with progression. **E.** RNA Expression of major EMT markers across all three parental cell lines. **F.** Example of raw image of scratch data at the 0-hour time point post scratch. **G.** Example of raw image of scratch data at the 24-hour time point post scratch.

Lastly, we used RT-PCR to confirm the expression of several EMT targets in our cell lines model the trends seen in previous EMT studies [24]. Indeed, our cell lines show a decrease in the epithelial marker E-Cadherin and an increase in mesenchymal markers N-Cadherin, Vimentin and MMP2 as we progress through the three cell lines in our model from benign to tumorigenic (**Figure 2.1.E**). E-Cadherin is at its highest mRNA expression in the MCF10A line, but its expression is more than halved in the MCF10AT1 cell line. The MCF10Ca1h cell line had the lowest expression of E-Cadherin which is to be expected due to the MCF10Ca1h cell line being metastatic. There was also a switch from E-Cadherin to N-Cadherin as N-Cadherin showed an increase as the model progressed from the MCF10A to the MCF10AT1 cell lines and subsequently to the MCF10Ca1h cell line. Interestingly, Vimentin and MMP2 did not show much difference between the MCF10A and MCF10AT1 cell lines but increased dramatically in the MCF10Ca1h cell lines. Raw scratch images at the time of the scratch,  $t = 0$  hours, as well as 24 hours after along with the measurements are shown in **Figure 2.1.F** and **Figure 2.1.G**.

Overall, our breast cancer progression model showed an increase in proliferation and migration between the MCF10A and MCF10AT1 Cell lines, as well as between the MCF10AT1 and MCF10Ca1h cell lines. Interestingly, the m6A levels were highest in the MCF10AT1 cell line followed by the MCF10Ca1h cell line and lowest in the MCF10A cell line. As expected, our epithelia markers were highest in our MCF10A cell line and lowest in our MCF10Ca1h cell line and inversely our mesenchymal markers were highest in the MCFH10CA1AH cell line and lowest in our MCF10A cell line. Having confirmed our model of breast cancer progression through EMT we next investigated the effect of decreasing m6A levels on that process.

#### *Knocking Down METTL3 and its effect on phenotype*

To investigate the role of m6A in breast cancer progression we knocked down METTL3 in our cell lines via transient CRISPR transfection targeting Exon 1 which was expected to cause a premature stop codon in the resulting mRNA. The plasmid used for the transient CRISPR transfection also expressed an

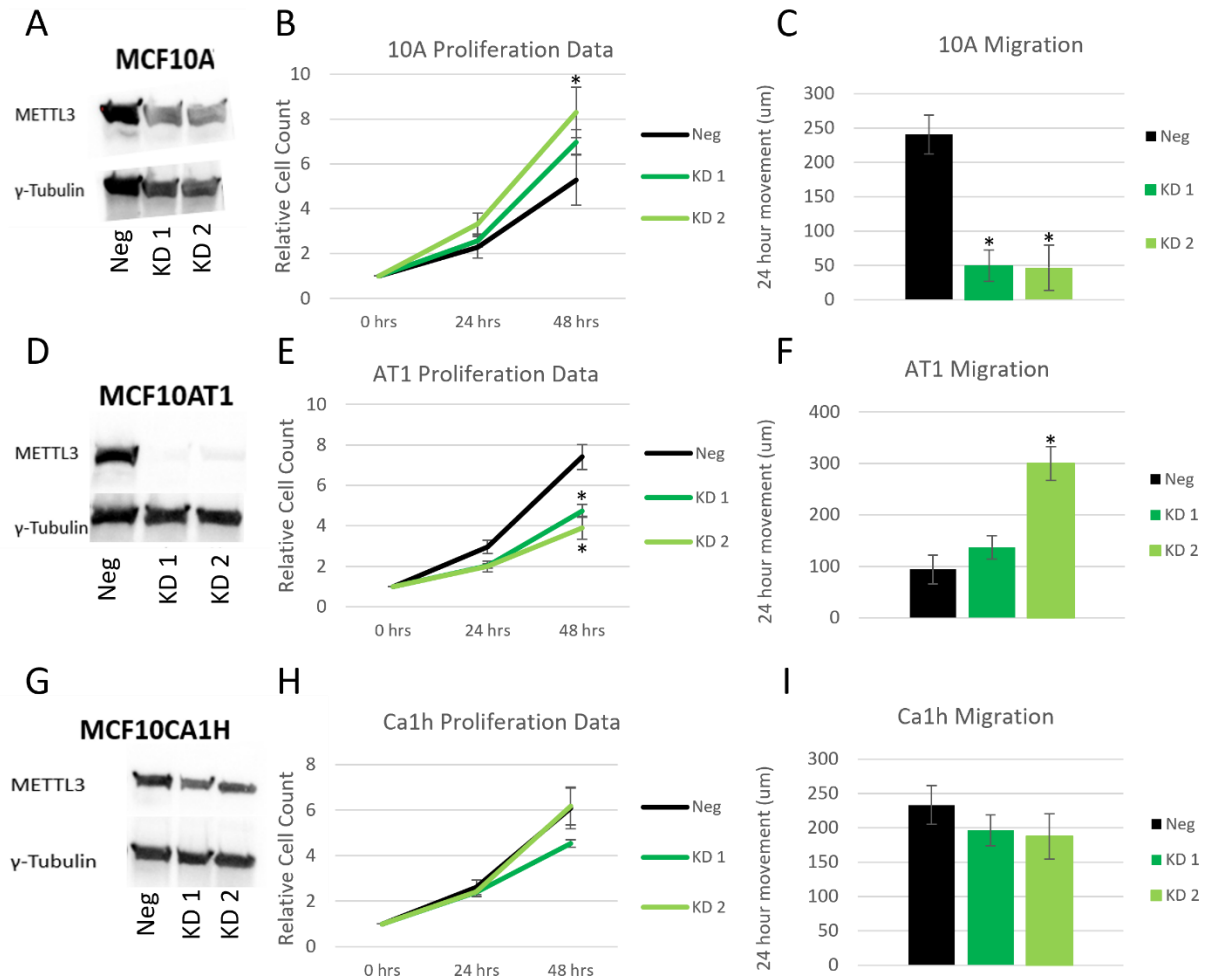
mCherry fluorescent protein that allowed FACS to be performed on the transfected cells. Transfection and FACS were performed twice followed by limiting dilution after which single cell candidates were cultured into stable cell lines. First and Second FACS of the MCF10CA1H Neg Crispr and Mettl3 Knockdown are shown in **Appendix 1** and **Appendix 2**, respectively. The knockdown of METTL3 was confirmed in each cell line via Western blotting and two candidates chosen for each line for use for this study. (**Figure 2.2A-2.2C**). In the MCF10A cell line, we observed knockdown in both our candidates with the second candidate having a more apparent knockdown. In the MCF10AT1 cell line we observed substantial knockdown in both cell lines. The MCF10Ca1h cell line proved to be the most resistant to METTL3 knockdown as evidenced by our best clones only having a moderate level of knockdown in both candidates.

We then followed up with proliferation and migration assays on the negative CRISPR that were also FACS sorted and the two selected knock down candidates on each of the three cell lines in our model. We observed that with the knockdown of METTL3 in the MCF10A cell line there was a significant increase in proliferation in one of the knockdown candidates (**Figure 2.2.D**) and a significant decrease in migration in both METTL3 knockdown lines compared to the negative CRISPR control (**Figure 2.2G**). In our proliferation assay we saw that our Negative CRISPR cell line doubled its cell count at the 24- and 48-hour time points while the one of the knockdowns nearly tripled at both time points. In our migration assay we saw that knocking down METTL3 in the MCF10A cell line decreased migration by nearly 80%. Interestingly, we saw the opposite effect in our MCF10AT1 cell lines. With the knockdown of METTL3, both knockdown lines of the MCF10AT1 cell line showed a significant decrease in proliferation (**Figure 2.2E**) and one knockdown line showed a significant increase in migration (**Figure 2.2H**). The proliferation rate with knockdown of METTL3 is nearly half the proliferation rate of the Negative CRISPR line. In our migration assay we observed that the distance travelled in our METTL3 knockdown line is three times more than the distance travelled by the Negative CRISPR cell line. This suggests that m6A via METTL3 has a dynamic role in the progression of our model and leads to unique changes in

phenotype based on the stage of the cancer. Our MCF10Ca1h cell lines showed no significant difference in proliferation (**Figure 2.2G**) or migration (**Figure 2.2.I**) between our negative CRISPR control and the two knockdown lines. This could be due to the fact that our knockdown candidates still had a fair amount of METTL3 present in the cells after our knockdown procedure (**Figure 2.2.C**). To summarize, the knockdown of METTL3 appeared to increased proliferation and decreased migration in the MCF10A cell line, while having the opposite effect in the MCF10AT1 cell line and no significant effect on the proliferation or migration of the MCF10Ca1h line suggesting that m6A plays differing roles in different stages of breast cancer development.

### *Changes in the transcriptome with the knockdown of METTL3*

Next, we performed a transcriptome wide analysis of our negative CRISPR cell line and one of the knockdown candidates for each cell line via RNA sequencing to determine which gene sets were most affected in our cell lines by the knockdown of METTL3. As mentioned previously, m6A is the most abundant post transcriptional modification found in eukaryotes, therefore we elected to focus on those mRNA targets which changed expression in response to METTL3 knockdown and could potentially be driving our phenotypic changes. Gene set enrichment analysis of the genes that showed significant differences between the control and METTL3 knockdown cell line was then performed for each cell line. In the MCF10A cell line, the cluster of genes with the most significant change in fold enrichment were Hallmark EMT genes (**Figure 2.3.A**). Genes in the Hallmark EMT cluster that had the most significant changes in fold enrichment included genes involved in Extracellular Matrix (ECM) and structure (ECS) organization, regulation of migration as well cell adhesion and motility. The MCF10AT1 cell line also showed the same cluster of Hallmark EMT genes having the most significant change in fold enrichment. (**Figure 2.3.B**) Of those genes, similarly to the MCF10A lines, the knockdown of METTL3 caused a significant change in cell motility and migration as well cell adhesion and ECM and ECS organization. Unlike the MCF10A cluster of genes, MCF10AT1 also showed a significant change in fold enrichment in



**Figure 2.2: m6A and its effect on phenotype.** A-C. Western Blots of 30ug of protein lysates from the MCF10A, MCF10AT1 and MCF10CA1H Cell lines with a single Negative Crispr line and two different Mettl3 Knockdown cell lines. (Representative of two experiments.) D-F. Proliferation cell count assays preformed on the previously mentioned cell lines. \* $P \leq 0.05$  by unpaired Student's t-test represents a statistically significant difference between the Negative Crispr cell line and the Mettl3 Knock down cell lines. Error bars represent SEM of 5 experiments. G-I. Migration/Scratch assays preformed on the previously mentioned cell lines with measurements taken at the time of the scratch and 24 hours after. \* $P \leq 0.05$  by unpaired Student's t-test represents a statistically significant difference between the Negative Crispr cell line and the Mettl3 Knock down cell lines. Error bars represent SEM of 5 experiments.

cornification and keratinization. This suggests that not only changes in cell motility and ECM-Cell interactions are involved in the increase in migration with the knockdown of METTL3. In the MCF10Ca1h cell lines, Hallmark EMT genes were the third most significant cluster of genes that saw a change in fold enrichment. (**Figure 2.3.C**) The two most affected gene clusters were Hallmark Interferon Alpha and Gamma response. These clusters in conjunction with the sixth gene cluster that saw the biggest change in fold enrichment, Hallmark P53 pathway, suggests a link between m6A via METTL3 and metastasized breast cancer's ability to avoid both the immune system and apoptosis.

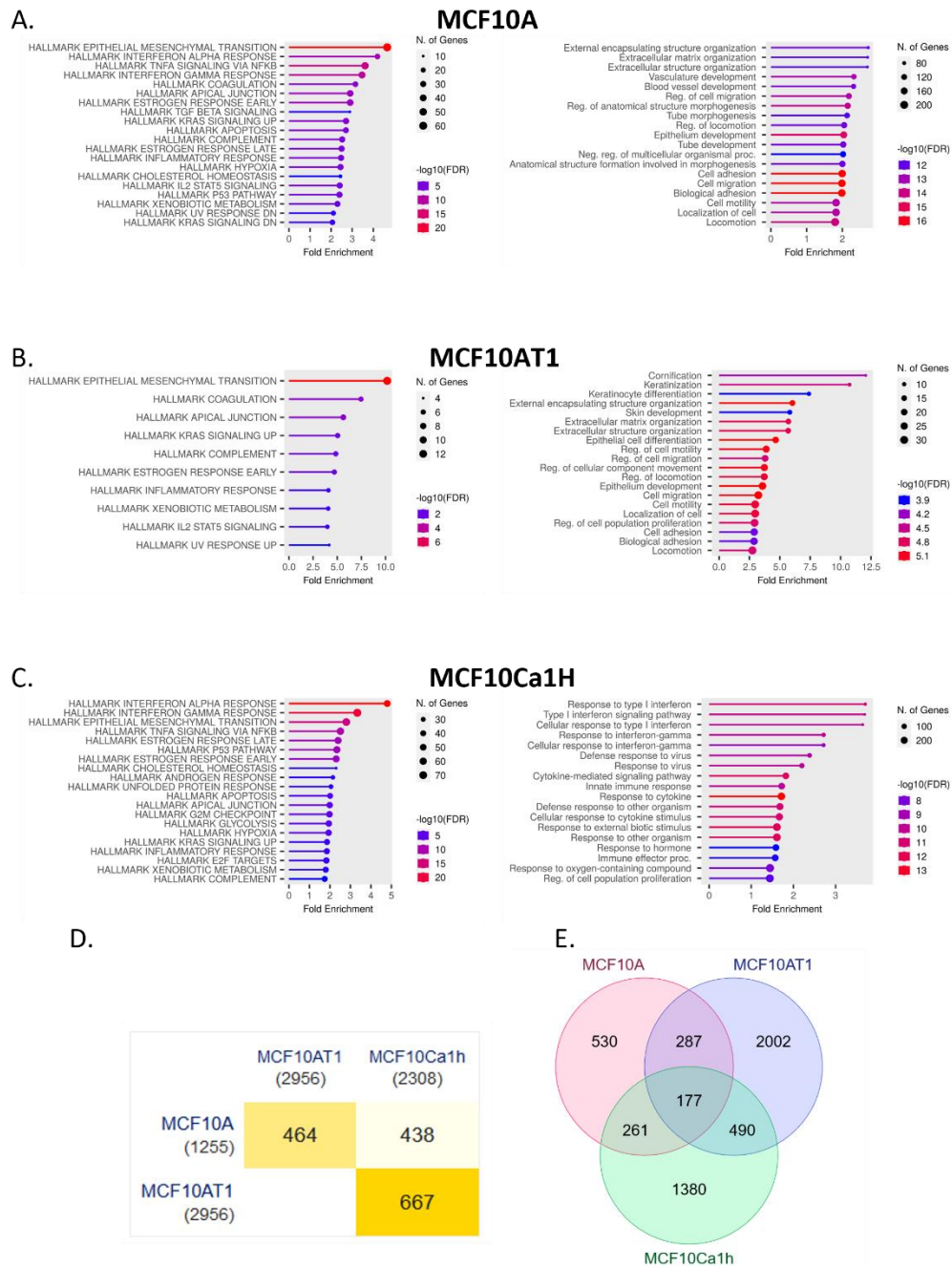
#### *Overlap of gene changes within the MCF10 Breast Cancer model with the Knockdown of METTL3*

We followed up our initial analysis of sequencing data with a comparison between the three cells lines of the significantly differing gene enrichment with the knockdown of METTL3. (**Figure 2.3.D**) Of the 1255 genes that showed a significant difference with the knockdown of METTL3 in the MFC10A cell line and the 2956 genes in the MCF10AT1 that showed a significant difference with the knockdown of METTL3, there was a 464 gene overlap (37.0% and 15.7% of the total for MCF10A and MCF10AT1 respectively.) Similarly, between the MCF10A and MCF10Ca1h cell lines there was a 438 significant gene change overlap (35.0% and 19.0% of the total for MCF10A and MCF10Ca1h respectively.) Interestingly, the most overlap of significant gene changes was between the MCF10AT1 and MCF10Ca1h with a 667 gene overlap (22.6% and 28.9% of the total for MCF10AT1 and MFC10Ca1h respectively.) 177 genes were found to overlap between all 3 cell lines with the knockdown of METTL3. Of those 177 genes, the largest cluster within this gene set belonged to Hallmark EMT genes (**Figure 2.3.E**). This suggests the m6A via METTL3 plays a significant role in mRNA regulation of EMT messages in breast cancer at all stages.

#### *M6A levels with the knockdown of METTL3 in Breast Cancer*



Next, we wanted to quantify our m6A levels in our cell lines with and without the knockdown of METTL3. Though it should be expected that m6A will decrease with the knockdown of METTL3, we wanted to confirm and quantify these changes in our cell lines. We did this by using a colorimetric EpiQuik m6A RNA Methylation Quantification Kit on total RNA for each of our cell lines in duplicate of two biological replicates. We saw a decrease in m6A in our MCF10A cell lines, but it was not statistically significant (**Figure 2.4A**). This may be due to the overall low level of m6A in the MCF10A cell lines. (**Figure 2.1.B**). In both our MCF10AT1 and MCF10Ca1h we saw a significant decrease in m6A levels in both our knockdown candidates compared to our negative CRISPR (**Figure 2.4C, 2.4E**). Interestingly, even with an unsubstantial knockdown of METTL3 in the MCF10Ca1h line (**Figure 2.2.G**) we still saw a significant drop in m6A levels compared to the negative CRISPR cell line. This data suggests that the changes seen in the transcriptome wide analysis most likely occurred due to m6A levels decreasing.

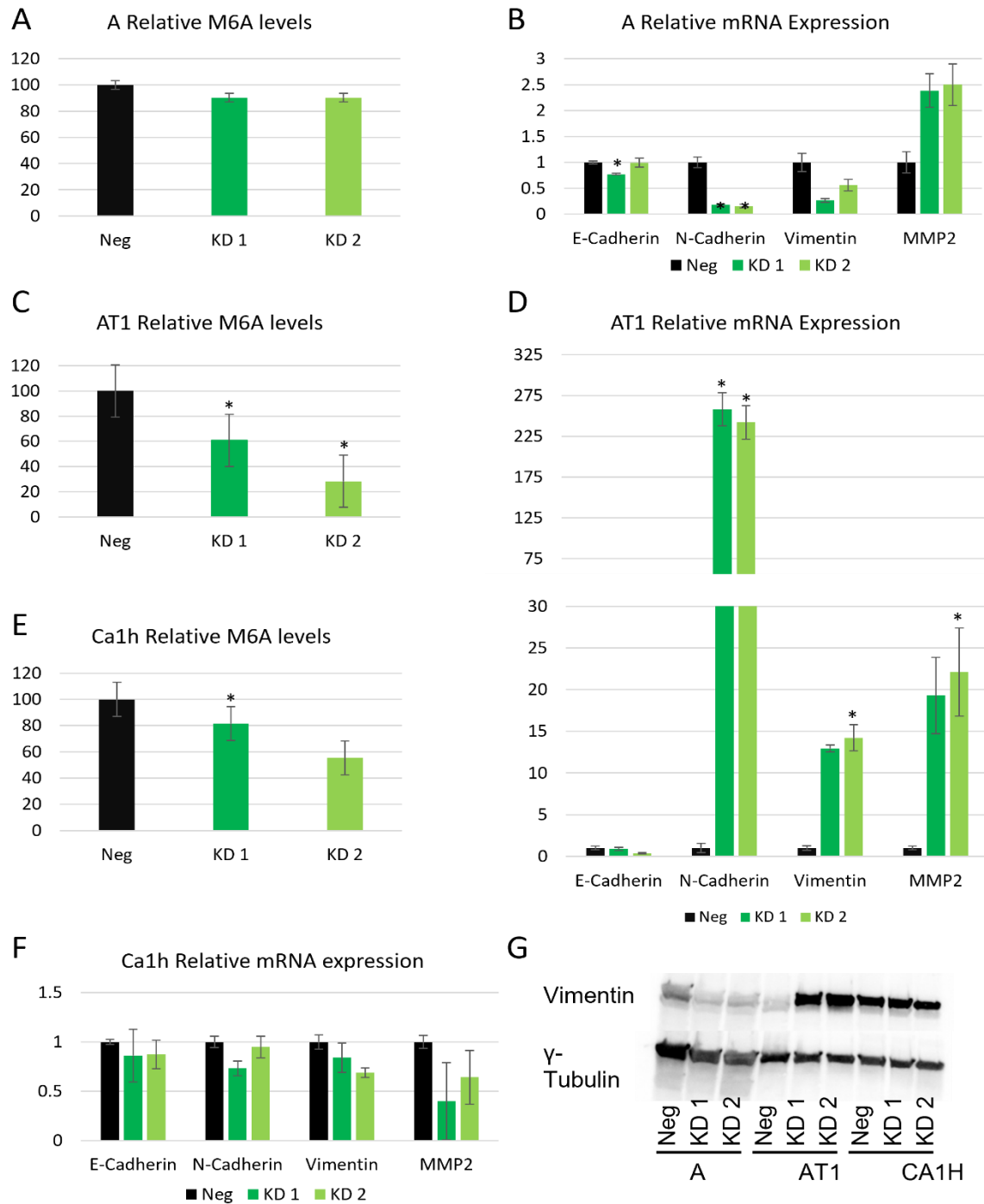


**Figure 2.3: Transcriptomics of Mettl3 Knockdown in the MCF10 Breast Cancer Model.** **A-C.** Gene set enrichment analysis of the genes significantly different in the Mettl3 CRISPR lines are compared to Control CRISPR lines for MCF10A, MCF10AT1, and MCF10Ca1H lines respectively. Left: Enrichment charts of the significant gene sets ranked by fold enrichment for the Human MSigDB Hallmarks gene collections Right: Enrichment charts of the significant gene sets ranked by fold enrichment for the Gene Ontology (GO) Biological Processes gene collections. All analysis was performed with ShinyGo with an FDR cutoff of 0.05. **D.** Overlap of all genes significantly different from Control in the three cell lines. Numbers in (parenthesis) are total number of significant genes in each line. **E.** Gene set enrichment analysis of the 177 genes showing significant changes in all three cell lines. Left: Enrichment charts of the significant gene sets ranked by fold enrichment for the Human MSigDB Hallmarks gene collections Right: : Enrichment charts of the significant gene sets ranked by fold enrichment for the Gene Ontology (GO) Biological Processes gene collections. Data sets were acquired from 3 biological replicates of the negative crisper cell line and a single Mettl3 knockdown cell line.

### *EMT gene expression*

As the EMT pathway was the hallmark most significantly affected by METTL3 knockdown in two of our cell lines (and third in the other) we wanted to further investigate changes in expression of several EMT targets with the knockdown of METTL3 at different stages. In the MCF10A cell lines, which typically express high levels of epithelial markers, we observed no change in mRNA expression of E-Cadherin with METTL3 knockdown and instead saw a decrease in N-Cadherin and Vimentin. N-Cadherin decreased about six-fold in both knockdowns while Vimentin decreased two to four-fold. Conversely, MMP2 saw a two and half fold increase in both knockdowns of METTL3 in the MCF10A line (**Figure 2.4.B**). For the MCF10AT1 line, we observed an increase in N-Cadherin, MMP2 and Vimentin in both our METTL3 Knockdowns of MCF10AT1 cell line and a decrease in E-cadherin in one of our knockdowns of METTL3 consistent with a switch to a mesenchymal phenotype (**Figure 2.4.D**). Vimentin was observed to have thirteen times more expression with the knockdown of METTL3 compared to the wild type while MMP2 expression increased about 20-fold with the knockdown of METTL3. Interestingly, N-Cadherin showed a substantial increase of about 250-fold with the knockdown of METTL3 compared to the Neg CRISPR in both of our cell lines. Interestingly, similar to the proliferation and migration results, the METTL3 knockdown in the MCF10Ca1h cell line did not show any significant effect on mRNA expression of several EMT targets (**Figure 2.4.F**).

With Vimentin emerging more and more as a center piece of EMT [3] as well as being likely the key player in the effects on migration we observed in response to METTL3 knockdown, we also determined Vimentin protein levels via Western blot. We found Vimentin protein levels followed the same trend as the mRNA expression across our three cell lines (**Figure 2.4.G**) and in response to METTL3 knockdown. This substantiating evidence suggests that Vimentin is likely the target of m6A regulation in regard to cell migration.



**Figure 2.4 mRNA and Protein Expression Changes with the Knockdown of Mettl3 in the MCF10 Breast Cancer Model** **A,C,E.** relative m6A quantification from the MCF10A, MCF10AT1, and MCF10CA1H cell lines. \* $P \leq 0.05$  by unpaired Student's t-test represents a statistically significant difference between the Negative Crispr cell line and the Mettl3 Knock down cell lines. Error bars represent SEM of 2 experiments **B,D,F.** Relative mRNA expression levels of EMT targets via RT-PCR \* $P \leq 0.05$  by unpaired Student's t-test represents a statistically significant difference between the Negative Crispr cell line and the Mettl3 Knock down cell lines. Error bars represent SEM of 3 experiments. **G** Western Blots of 30ug of protein lysates from the MCF10A, MCF10AT1 and MCF10CA1H Cell lines with a single Negative Crispr line and two different Mettl3 Knockdown cell lines. (Representative of two experiments.)

## Discussion

The exact role of m6A in cancer is still emerging. As mentioned previously, there is a plethora of data suggesting it both promotes and suppresses cancer [6,10-26]. However, this controversy in the literature gives credence to the fact that m6A does not have a static role in cancer progression. Although EMT and M6A are both highly researched topics, the link between the two is still emerging [8,23,25]. It is imperative to both determine m6A's dynamic role in cancer progression and how it is involved in EMT as it the most abundant mRNA modification in eukaryotes.

In this study, we show that m6A has a more dynamic role in cancer progression than previously thought. By using the well-defined MCF10 breast cancer model, we observed a diverse regulatory mechanism that adjusts in accordance with the stage of breast cancer progression. Our results provide a more detailed view of how m6A plays a unique role in proliferation as well as migration that can vary throughout EMT in breast cancer cells.

In the spontaneously immortalized breast cancer cells (MCF10A), lowering m6A by knocking down METTL3 caused the cells to become significantly more proliferative. This simultaneously caused the cells to become significantly less migratory as well. Similar results in migration have been shown in a previous study [31]. These results also mimic another study showing that proliferation and migration work as a tradeoff with each other as the cancer progresses [29]. This tradeoff may help explain why METTL3 knockdown in these cells would have affected cell proliferation over migration. In that study, it was shown that selection between proliferation and migration varied based on location within the tumor, cell turnover rates, the nature of the trade off, and response to the environment. While this study was done in vitro, the cellular environment that we grew the cells in might have favored proliferation over migration due to the abundance of nutrients available. Furthermore, as the MCF10A cell line is neither

transformed nor malignant it is likely the least capable of adjusting to changes compared to the other cell lines in this model.

The transformed MCF10AT1 cells came about by overexpression of an oncogenic version of HRas that results in constitutive activity. This small genetic change has large implications in both the cell phenotype as well as survival. We observed that this cell line had the highest transfection rate and the least amount of cell death from the FACS process (data not shown). These cells also showed an opposite effect in response to the decrease in m6A via knockdown of METTL3 in regard to proliferation and migration as compared to the MCF10A cell lines. Similar to other breast cancer studies, the proliferation rate significantly decreased [6,12], while the migration rate significantly increased [24], with METTL3 knockdown. This again supports the idea of a tradeoff the cells are committing to proliferation or migration. This also clearly shows that within a well-defined model, a small genetic change has caused the cancer cells to be transformed as well as tumorigenic has also caused the cells to utilize m6A in a different manner. Thus, in the presence of oncogenic G12V HRas, a decrease in m6A brings about an opposite phenotypical change in migration and proliferation as well as a down regulation of epithelial markers such as E-Cadherin and an upregulation of Mesenchymal markers such as N-Cadherin, MMP2 and Vimentin. Interestingly, we also saw a similar effect of G12V HRas altering the cellular response to changing m6A levels using a different model of breast cancer progression [13].

In the malignant cell line MCF10Ca1h, presumably large genomic changes occurred from the MCF10AT1 cell line during the serial mouse xenografts. This cell line is the most proliferative and migratory in the breast cancer model and represents advanced stages of disease. Interestingly enough it also had the least knockdown of METTL3 compared to the MCF10A and MCF10AT1 cell lines. While it is possible that this cell line is naturally resistant to CRISPR modification, it is more likely that the cells have become reliant on METTL3 and therefore resistant to its deletion. This potential resistance as well as the highly proliferative characteristic of these cells may be the reason for such minor change in METTL3.

Presuming METTL3 is required for MCF10Ca1h viability, if METTL3 levels drop below a certain level, the cell cannot survive. Thus, it is possible that the cells that did experience METTL3 knockdown simply died or were outcompeted by those that retained some level of METTL3 expression. The limited METTL3 knockdown obtained in the MCF10Ca1h cells had no significant effect on proliferation or migration. In addition to possibly not being a large enough knockdown to observe an effect (a theory made less likely by the fact that we did see gene expression changes) this resistance to change may be due to the malignant cells being at the upper limit of proliferation and migration rate. The MCF10Ca1h cells have a large number of genomic alterations in addition to the G12V HRas and represent a highly aggressive cancer cell. They are in the late stages of EMT and knocking down METTL3 may not be enough to push past those limits. If a better knockdown showed a similar yet more dramatic result, this could be further progression of cancer from EMT to Mesenchymal to Epithelial Transition (MET). MET has been shown to occur after metastasized cells have colonized other organs and tissues [31]. It would be interesting to repeat this study with a well-defined MET model to observe if the dynamic role of m6A shows similar results as this study.

We next investigated global changes in the transcriptome via RNA sequencing between our Negative CRISPR and METTL3 knock down cells in our breast cancer model to determine what pathways may be regulating the phenotypic changes observed. Interestingly, the cluster of genes that showed the most significant change in fold enrichment was Hallmark EMT genes in both the MCF10A and MCF10AT1 cell lines. More specifically, the genes within the EMT cluster were involved in both cell motility and migration as well as their regulation, locomotion, and cell adhesion. Uniquely, in the MCF10A cell lines, genes involved in vasculature and blood vessel development were affected with the knockdown of METTL3. This could be due to the fact that with the knockdown of METTL3 we observed an increase in proliferation. This proliferation could cause the cells to ignore the scaffolding of the extracellular matrix and in turn create a tumor that requires angiogenesis to maintain the cells within. In the MCF10AT1 cell line, within the gene cluster we observed the three most affected gene processes were cornification, keratinization, and keratinocyte differentiation. These gene processes were not observed to have changed

in the MCF10A and MCF10Ca1h cell lines. This could potentially explain why the METTL3 knockdown in the MCF10AT1 cell line had such a morphed shape compared to the negative control the MCF10A and MCF10Ca1h cell lines did not have an apparent effect on morphology with the knock down of METTL3 (data not shown).

The MCF10Ca1h cell line had the most unique gene processes affected by the knockdown of METTL3 with the top five gene processes affected all being related to type I interferon response and signaling. The following nine processes affected by our knockdown of METTL3 are all related to immune response to cytokines, viruses and external biotic stimuli. As mentioned before, if the metastasized MCF10Ca1h have reached the upper limit of migration and proliferation and as we observed in MCF10A and MCF10AT1 cell lines that m6A has a unique role at different stages, then this could answer why we did not see an effect on the phenotypes studied. m6A via METTL3 could have a much broader range of gene process regulation than previously thought. Future studies would be required to determine the mechanism of effect on these gene processes.

Finally, we wanted to determine the changes in expression of several EMT targets with the knockdown of METTL3. We observed that Vimentin, and N-Cadherin to lesser extent, was decreased in our knockdown of METTL3 in our MCF10A cell line. This coincides with our migration data that showed knocking out METTL3 decreases migration. Inversely, in the MCF10AT1 we showed an increase in Vimentin, N-Cadherin as well as MMP2 in our knockdown that also showed a significant increase in migration. We saw no significant changes on mRNA expression of our elected EMT targets in the MCF10Ca1h line with the knockdown of METTL3. Since we observed data that both Vimentin and N-Cadherin mRNA expression coincided with the trends seen in our migration data, we elected to run protein expression on Vimentin and that data gave more credence to the idea that Vimentin is the driving force for the changes in migration. This matches the literature that Vimentin has a key role in EMT [3]. Vimentin also plays a role in cell integrity and shape [32]. This could explain our change in morphology uniquely in our



MCF10AT1 cell lines. As more research is done on Vimentin and its role in EMT, this study strengthens the idea that Vimentin is at the center of EMT.

We have yet to determine the genes involved in the changes in proliferation in our MCF10A and MCF10AT1 cell lines. Since E-Cadherin also known as CDH1 is involved in both proliferation and contact inhibition we chose that gene for study but did not observe any substantial evidence that it alone could drive the changes in proliferation. If another gene or family of genes are to be studied, it would be the *CDK* family of genes. They showed significant changes in mRNA expression in our transcriptome wide analysis and have been shown to be involved in cell-cycle progression [30].

Further study of apoptosis and its relationship with m6A should be conducted as well in order to determine other regulatory gene pathways affected by m6A via METTL3. Through preliminary studies, we did not observe any large changes in cell death upon METTL3 depletion in the MCF10AT1 lines. We also attempted to see changes in the pro-caspase 9 and cleaved caspase 9 via western blotting as shown in **Appendix C** and observed no changes between the negative control lines and the METTL3 knockdown lines. However, the cells had grown for a number of weeks during after FACS sorting and were naturally selected for cells that grew well so the initial effects of METTL3 knockdown may have been missed. It has been shown in a previous study that the MCF10A line was resistant to p-methoxyphenyl p-toluenesulfonate (MPTS) with varying doses as well as time compared to other breast cancer cell lines such as MCF-7, MDA-MB-231, and MDA-MB-453, with mortality reaching only about 10% [33]. Other apoptosis inducing agents as well as optimization of the assay used must be tested in order to determine changes in apoptosis in our cell lines.

Overall, this study has shown that m6A via METTL3 has a very unique and dynamic role in EMT progression based the stage of the cancer in regard to proliferation and migration. The study also showed that the gene processes affected have some similarities across some the stages but not others and there are

very unique gene processes for each stage of breast cancer progression regulated by m6A. Lastly, Vimentin showed a significant change in both mRNA and protein expression that followed the same trend of change in migration in both the MCF10A and MCF10AT1 cell lines and to a lesser extent N-Cadherin mRNA expression as well.

## Methods

### Cell lines

The MCF10A, MCF10AT1 and MCF10Ca1h cell lines were obtained from the Karmanos Cancer center and maintained in DMEM (Dulbecco's Modified Eagle Medium) (Corning) and supplemented with 5% Horse serum along with 1% Penn/Strep, 0.2% Sodium Chloride, Insulin and Hydrocortisone respectively as well as 0.07% Cholera toxin and 0.04% Epidermal Growth Factor. These cells were passaged when reaching approximately 85-95% confluency. Cells were maintained on 10 cm dishes (CytoOne, USA Scientific, Orlando, FL) and experiments were run on 6 well plates. Cells were sustained in 37 ° C Incubator at 5% CO<sub>2</sub>.

### Knockdown of METTL3

Knockdown of METTL3 was accomplished by using CRISPR/Cas9 targeting EXON 1 with a deletion causing a frameshift resulting in a non-functional protein. The plasmid used also included an mCherry sequence. The CRISPR/Cas9/sgRNA all-in-one construct was transfected into all three cell lines using Lipofectamine 3000 (Thermo Fisher) according to manufactures directions. The cells were given 48 hours to recover after transfection with fresh media and prepared for Fluorescence Activated Cell Sorting (FACS). After cells had developed into a stable cell line the transfection and subsequent FACS sorting was repeated. The FACS began with cells without either of the plasmids as a negative control of the sort. The FACS included determining cells that were found along the path of the laser known as Forward Scatter Cells (FSC) and cells that were found at a ninety-degree angle of the laser known as Side Scatter Cells (SSC) followed by sorting cells that were found in the both the FSC and SSC plots as well as containing the mCherry fluorescence. After the second FACS sorting the cells developed into a stable cell

line and were diluted down to single cell per well and grown in a 96 well plate. Prime candidates were tracked and moved to subsequent plates when reaching ~80-90% confluency (24 well to 6 well to 10 cm dishes). Prime Candidate cell lines had their protein extracted and Western Blotting was used to confirm Knockdown of METTL3.

#### RNA extraction

Trizol (Life Technologies, Carlsbad, CA) was used for all RNA extractions according to the manufacturer's protocol. RNA was further purified and treated with RNase-Free DNase I (Life Technologies) using PureLink RNA Mini Kit (Life Technologies). RNA purity and quantity was determined via NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA).

#### LC-MS/MS and Sequencing of PolyA<sup>+</sup> RNA

PolyA<sup>+</sup> RNA was first purified from total RNA through oligo-dT selection using a Poly(A)Purist-MAG magnetic mRNA Purification Kit (Life Technologies) followed by ribosomal RNA depletion using RiboMinus Eukaryote Kit (Life Technologies) according to the manufacturer's protocols. Purified PolyA<sup>+</sup> RNA was digested to individual nucleosides and modified nucleosides were quantified as previously described [50]. Briefly, digestion was performed with nuclease P1 (Sigma, 2 U) in buffer containing 25 mM NaCl and 2.5 mM ZnCl<sub>2</sub> for 2 h at 37° C, followed by incubation with Antarctic Phosphatase (NEB, 5 U) for an additional 2 h at 37° C. Nucleosides were then separated and quantified using UPLC-MS/MS as previously described [51], except acetic acid replaced formic acid in the mobile phase. For sequencing 1ng in 30 ul of sample with water was sent for analysis. This was accomplished in collaboration with Dr. Huang and the ECU sequencing core.

## m6A Quantification

EpiQuik m6A RNA Methylation Quantification Kit (Colorimetric) was used to quantify relative m6A levels according to the manufactures protocol. 400 ng of total RNA was used. The colorimetric quantification was read by Promega Glomax Multiplus Plate Reader at 450 nm. Data was calculated using the equation determined by the manufacturer.

## Western Blotting

Whole cell lysates were prepared in whole cell extract buffer (WCEB: 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, and complete protease inhibitor (Promega, Madison, WI)). Equal amounts of protein (30 µg) were electrophoresed on a mini-PROTEAN any KD acrylamide gel (Bio-Rad Laboratories, Hercules, CA) and transferred to Hybond ECL nitrocellulose (GE Healthcare, Chicago, IL). The blot was blocked with 5% nonfat dry milk (LabScientific, Highlands, NJ) in Tris buffered saline with 0.1% Tween 20 (TBST) for one hour at room temperature, followed by primary antibody in blocking buffer overnight at 4° C. After washing extensively with TBST, blots were incubated for 1–2 hours at room temperature with appropriate anti-mouse (GE Healthcare), anti-rabbit or (GE Healthcare, washed again with TBST, detected using Bio-Rad Clarity Western ECL Substrate (Bio-Rad Laboratories), and imaged via Invitrogen Ibright FL1500 imaging system.

## Proliferation

Approximately  $2.5 \times 10^5$  cells were plated in to each well of a 6 well plate. After 24 hours from plating 2 wells were lifted with trypsin and counted via trypan blue and hemocytometer in duplicate. This process was done subsequently at the 48- and 72-hour time points. At each time point the wells that were not counted had been given fresh media to avoid nutrient/additive deficiency during the time frame of the experiment and to allow for constant proliferation.

## Migration

Approximately  $2.0 \times 10^6$ ,  $1.5 \times 10^6$ , and  $1.0 \times 10^6$  cells were plated for the MCF10A, MCF10AT1 and MCF10Ca1h cell line respectively in a 6 well plate. 24 hours after plating or when the cells reach 95-100% confluency the cells were given serum free media for 4 hours prior to the scratch. Afterwards, a scratch was made with a p20 pipette tip (USA Scientific), and cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Corning) and fresh serum free media added. Pictures were taken at 0 and 24 hours and wound healing determined by measuring the change in distance between the cells on both sides of the scratch. In order to ensure the pictures at both the 0 and 24 hour time point were at the exact location, the plates were marked on the bottom and the images taken were at the precise location that the markings were out of frame. Plates were saved up to 48 hours of the scratch in order to ensure full closure of the scratch had occurred.

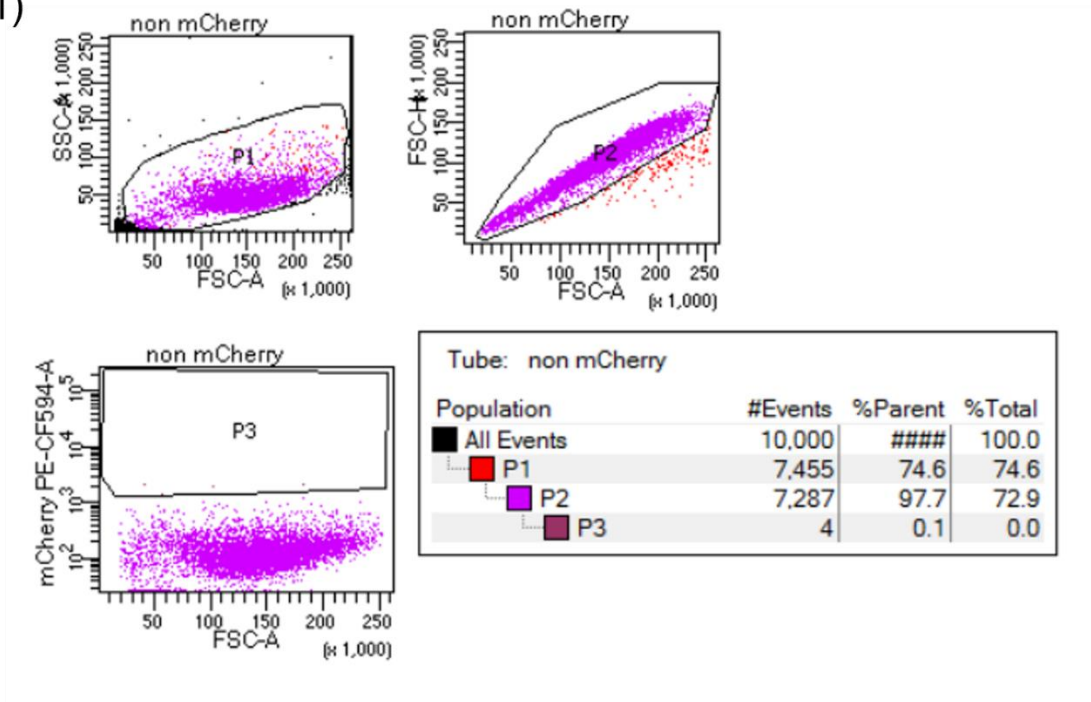
## RT-PCR

Reverse transcription was performed on 1 µg of total RNA in a 20 µl reaction with the iScript cDNA synthesis kit (Bio-Rad Laboratories, 170–8891). Quantitative real-time PCR was performed using a Roche Lightcycler 96 with Fast Start Essential DNA Green (Roche Diagnostics Corporation, 06-924-204-001) and primers from Integrated DNA Technologies, Inc. Primer efficiency was verified to be over 95% for all primer sets used. Quantification of mRNA was carried out via  $\Delta\Delta CT$  analysis using 18S rRNA and the respective control condition for normalization. All real-time PCR primer sets were designed so the products would span at least one intron (>1kb when possible) to prevent detection of the pre-mRNA and/or DNA, and amplification of a single product was confirmed melting curve analysis

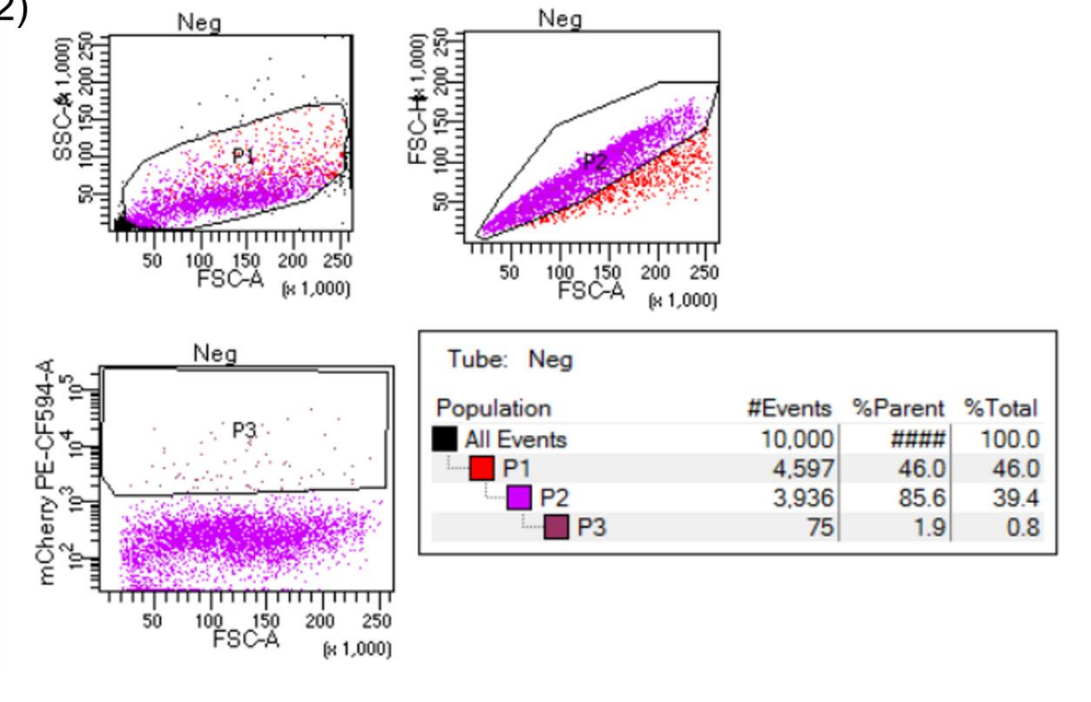
Appendices

Appendix A

(1)

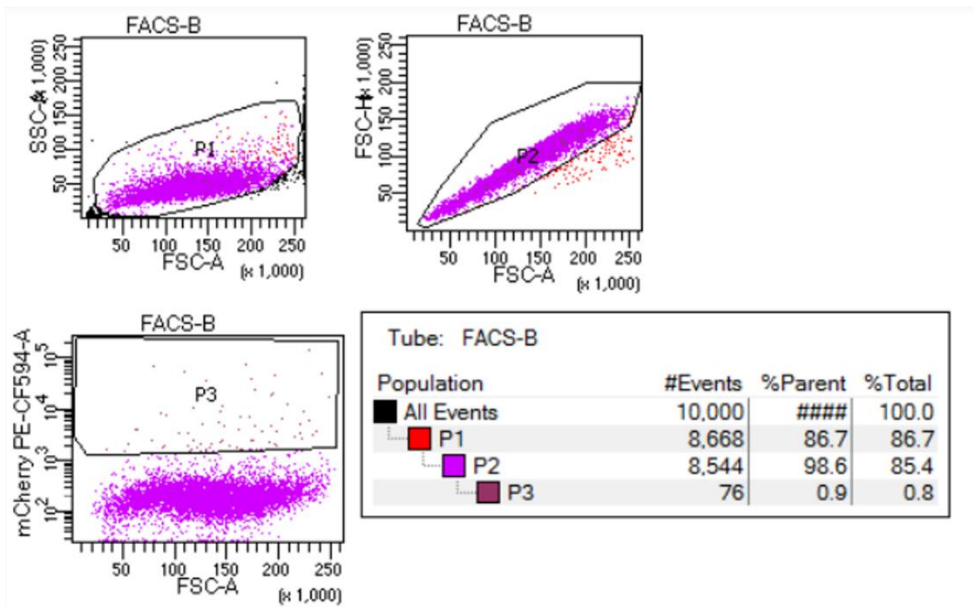


(2)

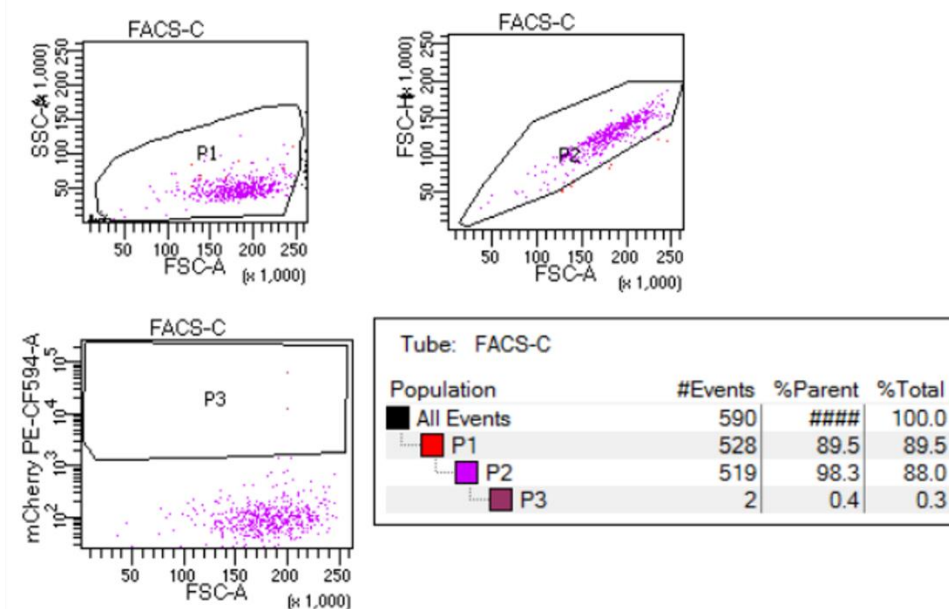




(3)



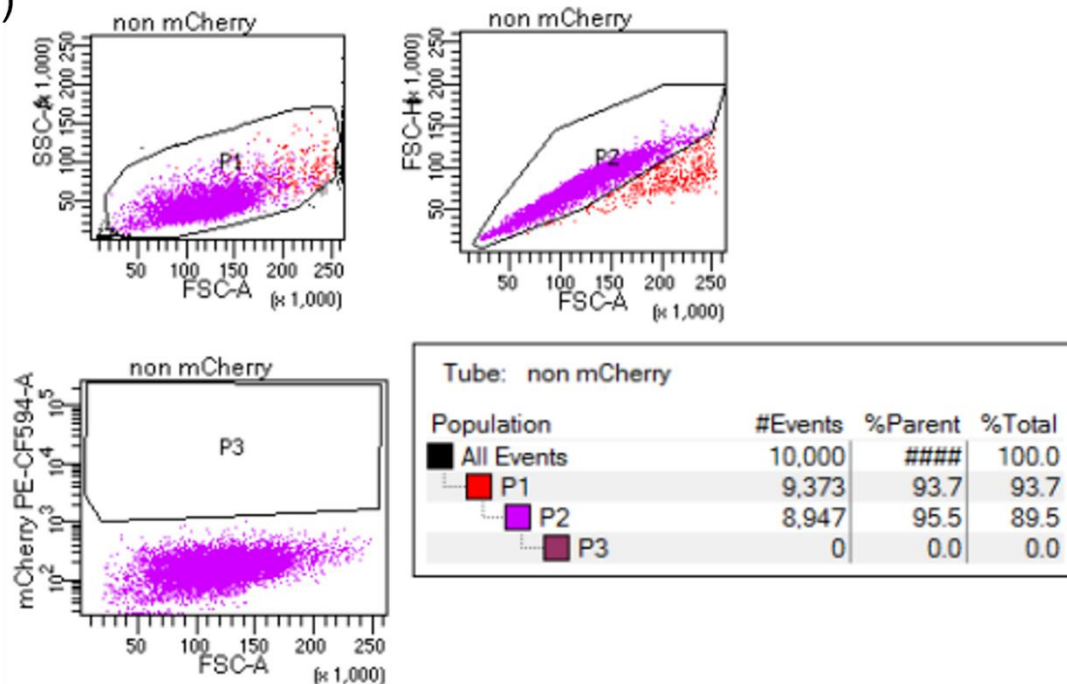
(4)



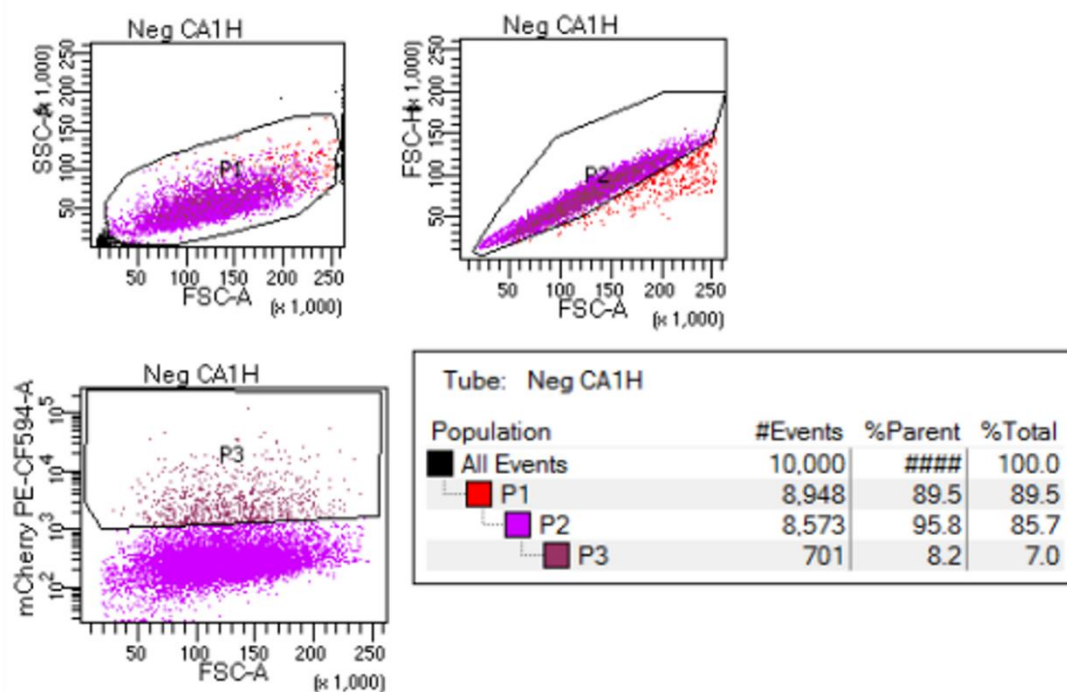
**Appendix A: First FACS of Neg Crispr and METTL3 Knockdown in the MCF10Ca1H Lines. A.1.** FACS gating of non transfected MCF10Ca1H cells. **A.2** FACS gating of MCF10Ca1H cells transfected with the negative Crispr plasmid. **A.3-A.4.** FACS gating of MCF10Ca1H of two populations of cells transfected with the Mettl3 Crispr plasmid. Gating was accomplished by first determining cells found along the path of the laser (Forward Scatter Cells also known as FSC) followed by cells found at a ninety-degree angle to the laser (Side Scatter Cells also known as SSC) and lastly sorting the cells with the mCherry fluorescence from the population of cells in both FSC and SSC plots.

## Appendix B

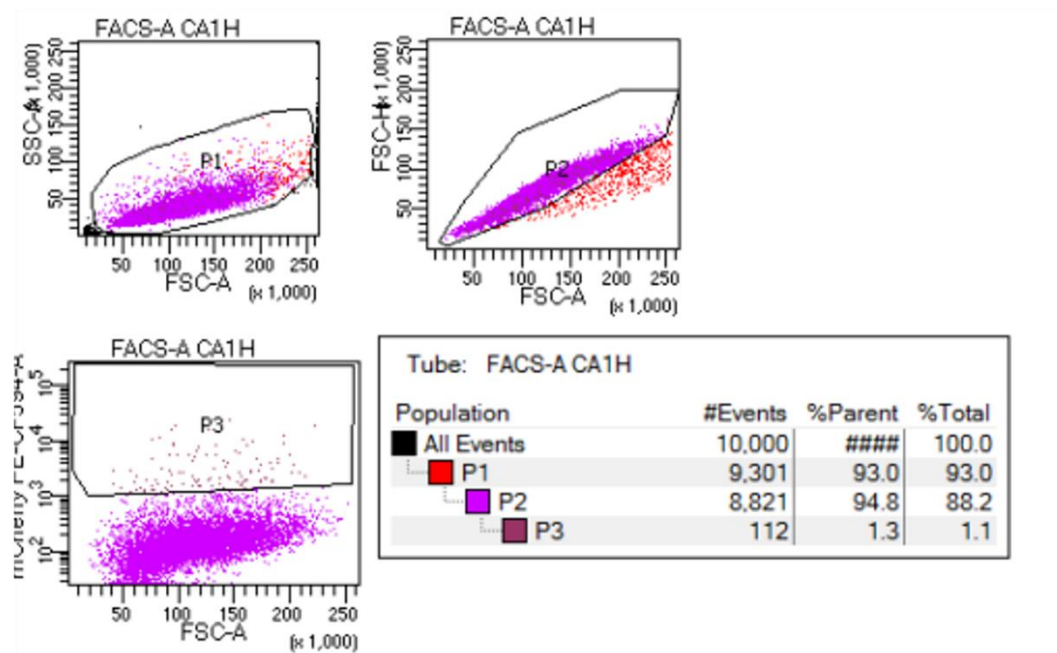
(1)



(2)

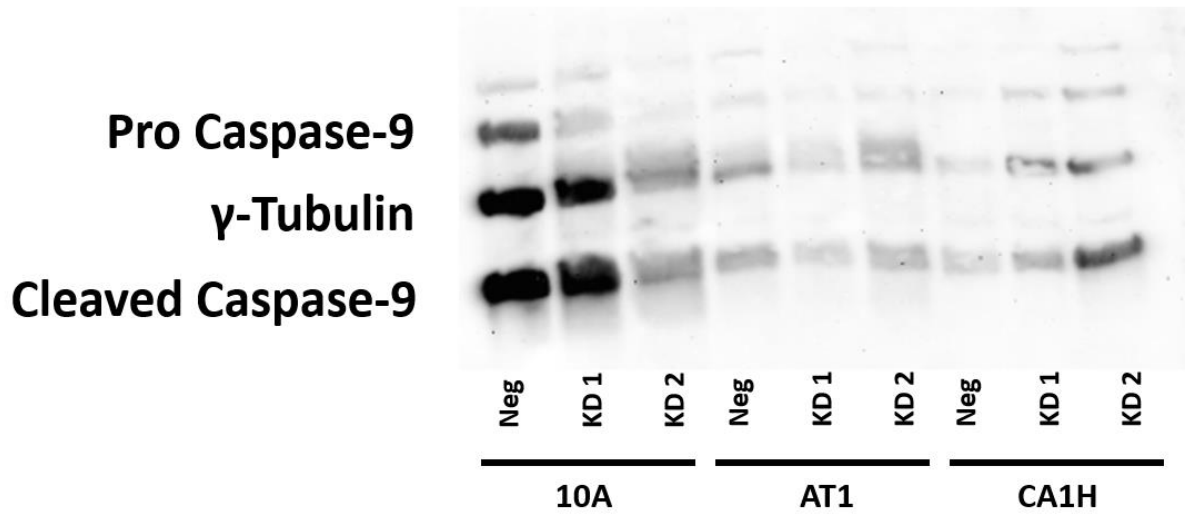


(3)



**Appendix B: Second FACS of Neg Crispr and METTL3 Knockdown in the MCF10Ca1H Lines. B.1.** FACS gating of non transfected MCF10Ca1H cells. **B.2** FACS gating of MCF10Ca1H cells transfected with the negative Crispr plasmid. **B.3.** FACS gating of MCF10Ca1H of cells transfected with the Mettl3 Crispr plasmid. Gating was accomplished by first determining cells found along the path of the laser (Forward Scatter Cells also known as FSC) followed by cells found at a ninety-degree angle to the laser (Side Scatter Cells also known as SSC) and lastly sorting the cells with the mCherry fluorescence from the population of cells in both FSC and SSC plots.

## Appendix C



### Appendix C: Western Blot of Caspase 9 in all three cell lines with METTL3 Knockdown.

Western Blot of 30ug lysates from the MCF10A, MCF10AT1 and MCF10Ca1H cell lines with a single Negative Crispr line and two different Mettl3 Knockdown cell lines.

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## **Chapter 3: Discussion**

## Introduction

Currently, there are many studies that attempt to characterize the role of m6A in cancer progression. Unfortunately, within this plethora of data, a consensus has yet to be met. Depending on cancer type, m6A player manipulated, the phenotype of the cancer studied, as well as the gene targets analyzed, the results are still inconclusive and at times conflicting. To address this, we hypothesized that a possible reason for this controversy in the field is that stage of the cancer was not accounted for. It is known that cancer adapts based on the micro-environment surrounding the cells and that cancer has progressed through distinct stages in a process known as type 3 Epithelial to Mesenchymal Transition (EMT). In order to test this hypothesis, we first needed to develop a breast cancer model that would allow us to determine the role of m6A in different stages of the same cancer and determine if stage could indeed influence the impact of m6A.

## Justifications

### *MCF10 model*

There are nearly 100 different breast cancer cell lines which makes choosing one to study a difficult task. Indeed, random investigation of disparate cell lines has led to the previously mentioned conflicting data within the m6A field in general but more specifically about the role of m6A in breast cancer. We set out in this study to address that conflict and so choosing a suitable model was an important consideration. The MCF10 model of breast cancer progression was developed by the Karmanos Cancer Center at Wayne State University in 1984. The MCF10 cell line is derived from benign proliferative breast tissue extracted from a Caucasian female that spontaneously immortalized in culture. For our MCF10 model, we chose three cell lines at different stages of progression. The MCF10As, which are the original spontaneously

immortalized MCF10 cells, are early-stage cancer cells that have a basal-like cell structure [1]. They do not express estrogen receptors and are not tumorigenic [2]. These represent very early-stage cancer cells that are our starting point in understanding the regulation required for breast cancer progression. We have designated these cells to mimic Stage 0-1 breast cancer cells. The next line of cells in the progression are the MCF10AT1s which are slightly more proliferative as well as migratory than the MCF10A's. This is due to a single genetic change of HRas G12V mutation that occurred via transfection. These cells were selected to mimic Stage 2-3 breast cancer cells. Last are the MCF10Ca1h's, which are metastasized cells that are extremely migratory, invasive, and proliferative. They were formulated via serial mouse xenografts of the MCF10AT1 cells that were injected into the mammary fat pad in mice and led to high genomic instability as well as several copy number gain and loss mutations as they continued to grow. The MCF10Ca1h cells were chosen to mimic Stage 4 breast cancer cells. We opted to use this model because it is widely used to study cancer progression as well as EMT [2,3]. We also used this model in order to explore a potential explanation of why so many groups are observing conflicting results when studying the role of m6A in cancer. Fortunately, we observed our hypothesis to be correct in that the role of m6A in breast cancer progression differs between our cell lines that were elected to represent different stages in breast cancer.

#### *Development of the METTL3 Knockdown Cell lines*

After determining the breast cancer model to be used for our experimentation, we began to knockdown METTL3 via CRISPR-Cas9 and gRNA to target exon 1 in order to cause a premature stop codon and an eventual truncated mRNA transcript. Also included in the plasmid was a mCherry fluorescent protein that allowed us to use FACS to attain a pure population of cells that had received the plasmid. The assumption is that cells that received the plasmid would produce mCherry fluorescence and would contain the gRNA as well as the CRISPR/Cas9 and then clones could be confirmed to have the deletion by Western Blotting to identify a decrease in METTL3 expression. Previous attempts at transfection were highly successful using the Lipofectamine 2000 product in HEK-293T cell lines with over 90% transfection rate determined

via microscopy. Unfortunately, that same protocol led to a less than one percent transfection rate in all three cell lines. The cells that contained the mCherry fluorescence were sorted and plated into 3 groups, (A-C) due to the low number of surviving cells in all three cell lines. Once the sorted cells had proliferated into stable cell lines, western blotting was done in order to determine the level of knockdown and two of the three groups in each cell line were used for subsequential transfection and FACS. This was also done with a Negative CRISPR control line to be used as the control for our further experimentation. This Negative CRISPR plasmid included the mCherry sequence in the plasmid as well as a control gRNA that was non-targeting and did not recognize any sequence in the human genome. This ensures the changes observed are not due to general effects of the CRISPR/Cas9 system expression. The subsequent transfection was done with Lipofectamine 3000 and FACS was again used in order to potentially have a stronger knockdown of METTL3. Unfortunately, the transfection rate increased minimally, less than five percent transfection rate. However, several groups of cells were isolated and allowed to proliferate into stable cell lines and were used in a Western blot to determine levels of knockdown of METTL3. The knockdown of METTL3 was more apparent after the second transfection and FACS but was still not enough to continue our experimentation. To isolate clones with better METTL3 knockdown, two prime candidates from each cell line's second transfection and FACS were diluted down into single cell wells and were grown over several months. From nearly a dozen potential candidates in each of the cell lines in our breast cancer model, two prime candidates were selected for experimentation after determining METTL3 protein expression levels via western blotting.

### *Phenotypic Assays*

In order to determine changes in phenotype with the knockdown of METTL3 and eventual attenuation of m6A in mRNA, we elected to study proliferation and migration. These two phenotypes were selected for several reasons. The first being that they are both major characteristics in the hallmarks/capabilities of cancer first published over two decades ago [41]. They are also some of the most studied Hallmarks in regards cancer progression as well as m6A. Another justification for choosing these two phenotypes to

study is that they have been shown to be at times antithetical to each other in that a switch is made in the cancer cells to prioritize one over the other [42]. This trade-off is likely due to dynamic microenvironments both within and around a tumor. A third justification is that as cancer progresses the capabilities and capacity of the cancer in regards to these two phenotypes is altered. In regards to our model we determined that proliferation and migration increase as the cancer progresses. Lastly, we elected these phenotypes over others such as tumorigenicity and metastasis due to the fact the our MCF10A lines are neither metastatic nor tumorigenic while the MCF10AT1 are not metastatic.

We then determined the best mode of testing based on both the equipment present as well as previous success in previous publications was to determine a relative cell count at 24 and 48 hour time points in order to study proliferation and a wound healing assay to determine migration over 24 hours. We also optimized our previous protocols to ensure the cells maintained a nutrient rich environment throughout the cell proliferation assay in order to ensure proliferation was not attenuated or halted due to nutrient deficiency. For our wound healing assay, we maintained a very precise measurement technique in order to limit variability in the data.

## **Contributions to the field**

The goal of this thesis is to begin a paradigm shift in our understanding of m6A's role in cancer. There are three directions I believe the field should be diverted to. The first is related to the specificity of the terminology used in published research. As mentioned previously, m6A is being described in a binary fashion in regards to its effect on cancer. It either promotes or suppresses cancer. Unfortunately, this is one of the major cruxes of the literature that is being convoluted. Promoting and suppressing cancer are overarching definitions that leave much to be discussed in regards to an already major and encompassing regulatory process, m6A. Currently, cancer has over a dozen hallmarks that have been shown to not only progress in an independent manner, but also have been shown to progress in exchange for another

hallmark being suppressed such as proliferation and migration [21]. Not all hallmarks have a dichotomy while others do progress in unison such as migration and invasion. This is why it is crucial when describing m6A and its dynamic role in cancer, to present the data with clear specificity in the terminology used. If this precision is not maintained, there is a high chance of misinterpreting or misunderstanding the data observed. For example, the data I presented in this thesis showed an increase in proliferation in the MFC10A cell line with the knockdown of METTL3 and a decrease in proliferation in the MCF10AT1 cell line with the knockdown of METTL3. If one were to take a myopic point of view of cancer being more proliferative as it progresses, a claim could be made that m6A via METTL3 suppresses cancer in MCF10A cell line due its decrease leading to more proliferation and m6A promotes cancer in the MCF10AT1 cell line due to its attenuation leading to less proliferation. If this same myopic point of view focused on migration, it would lead to the opposite conclusion based on the data presented in this thesis. We have presented a major cause of controversy in the field with the seemingly contradictory results with two cell lines that developed from a single parental cell line. This issue is then exacerbated and exponentially larger when hundreds of groups using thousands of different cell lines from over a dozen organs to study an already overarching regulatory mechanism. It is absolutely imperative in order to better understand m6A's role in cancer to be meticulous in the diction used to describe results that are observed. This will allow for better understanding across the field as well as lead to a consolidation of at times contradictory data while alleviating the controversy within the literature.

The second thing to take into consideration is the stage of the cancer being studied. We have presented a similar controversy to what is observed in the field in our data. We see differences in the response to m6A loss in not only the same cell type but between two cell lines that have the same origin, and the single difference between them is a single amino acid change in hRas that allows for it to be constitutively active. It has been known that cancer is highly adaptive to its microenvironment, so then why is it overlooked that it can use the most commonly found RNA modification for different biological processes based on need. We have shown that our model follows the previously observed trend of increased proliferation and

migration capacity as the cancer progresses. The MCF10A cell line is the least proliferative and migratory followed by the MCF10AT1 cell line compared to the MCF10Ca1h cell line. We have also shown that the cells utilize m6A in a unique way depending on the stage. In the MCF10A cell line as m6A decreased so did migration capacity while proliferation capacity increased while a decrease in m6A the MCF10AT1 cell line showed the opposite effect in that it was observed the MCF10AT1 cell line had a decrease in proliferation capacity and an increase in migratory capacity. A decrease in m6A in the MCF10Ca1h cell line showed no change in proliferation or migration capacity and transcriptomics showed a change in genes related to the immune response. This is why future studies looking to determine m6A's role in cancer progression must take into consideration that the role is dynamic between cell type as well as stage.

It is also known that as EMT progresses the cancer cells adjust based to necessity such as angiogenesis due to hypoxic conditions. These are clear indications that m6A is a multipurpose tool for cancer cells to utilize depending on the microenvironment the cells are in. In the transcriptomics data presented in this thesis, it is apparent that m6A is utilized to promote EMT in a unique way depending on stage. In the MCF10A cell line, the knockdown of METTL3 and eventual decrease in m6A affected genes related to EMT and more specifically genes related to extracellular matrix and structure organization. In the MCF10AT1 cell line, the expression of EMT genes most affected by the m6A attenuation were related to cornification and Keratinization. Though with the attenuation of m6A, both the MCF10A and MCF10AT1 showed differences in expression in genes related to cell migration, cell proliferation and cell adhesion, they lead to opposite effects in phenotype. The MCF10Ca1h cell line showed no change in phenotype and genes related to the immune response were most affected. The consideration of stage when determining the role of m6A in cancer progression as well as EMT and its effect on phenotype as well as gene regulation is necessary for better characterization and understanding of the unique and dynamic results presented in both this thesis and the field as a whole.

## Future Directions

In order to further characterize m6A's dynamic role in cancer, several phenotypical assays can be utilized. As mentioned previously in this thesis, escaping apoptosis in response to genomic damage is a hallmark of cancer. A link between m6A and apoptosis by a group showed that a decrease in m6A knockdown of METTL3 in MDA-MB-231 and MCF-7 breast cancer cells lead to an increase in apoptosis [4]. They also showed that the link is via targeting of the gene B-Cell Lymphoma 2, Bcl-2, that encodes for a protein that is found in the mitochondrial membrane that is responsible for preventing apoptosis by either preventing the release of mitochondrial apoptogenic factors such as cytochrome C and apoptosis-inducing factor, AIF, or by blocking the formation of caspases [5]. A different group showed similar results in HepG2 cells and showed a direct link with m6A and p53 [6]. Though we were unable to optimize our apoptosis assay via flow cytometry in our MCF10AT1 cell line and did not see changes in cleaved caspase-9 via western blotting. It is worth investigating if opposite results can be found depending on the stage in our breast cancer cell lines similar to our proliferation and migration data of our MCF10A and MCF10AT1 cell lines.

As mentioned before, another phenotypic assay that could also help characterize the breast cancer cell lines is an invasion assay. We did not prioritize optimizing this assay due to many papers showing parallel results in m6A's effect on migration and invasion in breast cancer [7,8]. The assumption is that if migration data will coincide with invasion data and will only minimally increase overall characterization of m6A's relationship with phenotype. Rather than focusing on experiments that have shown similar results to data that we have acquired, we believe that the best way forward is to determine m6A's effect on tumor size and weight in an animal model. Just like our MCF10Ca1h lines were developed by serial injection of MCF10AT1 cells in the mammary fat pad in mice, we would replicate that protocol with all three cell lines with and without our METTL3 knockdown. These tumors would be sized weekly and have their volume weighed after the mice are sacrificed to determine the effect m6A via knockdown of METTL3 has on tumor formation at different stages. This protocol for cell delivery was published by the



Zhang group at Capital Medical University in Beijing [9]. Some groups have shown that m6A suppresses tumor growth by studying the erasers FTO in breast tissue and ALKBH5 in Head and neck squamous cell carcinoma (HNSCC), while another group showed that m6A promotes tumor growth via attenuation of m6A writer KIAA1429 that is part of the METTL3- METTL14-WTAP complex [10-12]. As such, determining if our breast cancer model showed similar asymmetrical results in regard to m6A's effect on tumor size and volume depending on the stage.

Though there are over a dozen hallmarks of cancers that can be studied, in this study we have elected to focus the most relevant in terms of EMT and Breast cancer progression. If the results of these assays had been negative in that decreasing m6A via the knockdown of METTL3 shows no change in any of the several phenotypical assays discussed in this thesis, other phenotypical assays could have been performed to determine if m6A attenuates or augments the Warburg effect in our breast cancer model. During maintenance of the cells, we did notice a distinct color change in the media (likely due to a decreased pH with the production of lactic acid) occurred at a faster rate in the knockdown cells in the MCF10A and MCF10Ca1h cell lines. This suggests these cells may have been relying more on glycolysis than the wild-type lines and hence exhibiting the Warburg effect in response to the decrease in m6A.

Currently in the field, m6A has been shown to augment the Warburg effect in cervical, gastric, lung, pancreatic and breast cancer as well as attenuate the Warburg effect in pancreatic cancer and renal cell carcinoma [34-40]. m6A augments the Warburg effect in Cervical and Gastric cancers through enhancing the stability of the first important rate limiting enzyme in glycolysis, Hexokinase-2, that catalyzes the first committed step in glycolysis by phosphorylating glucose into Glucose-6-phosphate. In lung cancer, m6A enhanced MYC translation via m6A reader YTHDF1 that lead to an increase in glycolysis [36]. In pancreatic cancer, m6A on microRNA pri-miR-30d lead to degradation that allowed for an increase in expression of Hexokinase 1 (*HK1*) and Glucose transporter 1 (*GLUT1*). In breast cancer it was observed that m6A on Enolase 1 (*ENO1*) increased the transcript stability that allowed for higher expression and that lead to increased glycolysis [38]. ENO1 increases glycolysis by catalyzing the conversion of 2-

phosphoglycerate to phosphoenolpyruvate. It was also observed that m6A attenuated glycolysis in pancreatic cancer by promoting maturation of microRNA mir-30d that targets runt-related transcription factor (RUNX1) that regulates HK1 expression [39]. In renal cell carcinoma downregulation of METTL14 led to an accumulation of Bromodomain PHD finger transcription factor (*BPTF*) due to increased stability with the removal of m6A caused an increase in mRNA expression of ENO2 as well as an increase in glucose uptake and lactate production [40]. Overall glucose metabolism is an emerging direction to take in the field of m6A and cancer phenotype.

Another future direction to further the research topic is the m6A readers. There are several m6A writers that have different targets as well as different consensus sequences [22]. Due to having different gene targets as well as different molecule types such as mRNA and rRNA, the field mostly elected to modify m6A levels with manipulation of METTL3. As mentioned before METTL3 is responsible for catalytic activity in the METTL3- METTL14-WTAP complex. This is the major reason we targeted METTL3 in our study rather than METTL14 and WTAP. The m6A modification alone is not enough to induce a regulatory change and is also reversible via the m6A erasers. Two commonly studied m6A erasers ALKBH5 and FTO have been found to have distinct pathways and intermediates when demethylating their targets [23]. Though a consensus sequence is has not found for either m6A eraser, it has been observed that each eraser shows a distinct discrimination in the sequence of m6A targets they methylate [24]. Therefore, when manipulating these writers or erasers both an overall m6A level in mRNAs as well as target specific m6A analysis can be performed in order to have a more complete understanding of m6A and its role in cancer. These manipulations are merely a means to an end rather than the direct cause of changes observed in cancer progression.. There are steps further downstream in the molecular mechanism of m6A regulation. As mentioned previously, the m6A site alone does not induce a regulatory change but rather m6A readers such as the YTH family of proteins interact with the m6A modification on mRNA transcripts and then recruit their respective machinery to induce a regulatory change. These respective regulatory changes include mRNA stability, alternative, splicing, translation efficiency, nuclear export and

P-body storage [13,14]. Just as m6A writers and erasers are diverse in their targets based on both the molecule itself as well as the sequence of bases in the molecule, m6A readers are diverse in the mode of regulatory change they induce. One of the most studied families of m6A readers is the YTH family. YTHDC1 is involved in alternative splicing, YTHDF1 has been shown to be involved in translation efficiency and YTHDF2 plays a role in mRNA stability [13-15]. The phenotypical assays allow for the field to determine the biological processes and several targets that m6A is involved in but does not address the molecular mechanism that leads to the phenotypical changes observed. For example, YTHDF1 is involved in increasing translation efficiency while YTHDF2 is involved in recruiting machinery for decay of mRNA transcripts [25-29]. These two readers lead to opposite effects on protein expression. Potentially an m6A site could both promote and suppress mRNA and eventual protein expression of the same gene depending on which reader interacts with the m6A site. This expression difference can also change the promotion or suppression of a gene related to a specific hallmark of cancer and overall fate of a cancer cell. Unfortunately, direct correlations between cancer progression and specific m6A writers, erasers, readers and sometimes even m6A levels alone are being made without full grasping the complexity of the biological process of the RNA modification m6A. Just as the field observes different phenotypical changes based on the writer or eraser of interest that is involved in m6A, there is also a level of understanding of the reader that is required. For example, if an oncogene transcript is methylated with m6A and YTHDF1 is the reader that interacts with that methylation to allow for increased translation efficiency, this would result in an increase in expression of the oncogene and furthermore the progression of cancer. On the contrary, if YTHDF2 is the reader to interact with the methyl group and recruit's transcript degradation machinery, the oncogene will now have an overall decrease in expression and an overall suppression of cancer would occur. This added layer of experimentation can help alleviate the major issue of convoluted data observed in the field about m6A's role of both suppressing and promoting cancer.

With the transcriptomic sequencing data along with our RT-PCR data of changed mRNA messages with the decrease of m6A via the knockdown of METTL3 in our breast cancer model, we can follow up with an optimized m6A Immunoprecipitation that allows for us to determine if some of the mRNA expression changes are due directly to a decrease in m6A on that specific transcript. If we are unable to optimize this protocol for our goals or if it is no longer efficient to run Ips on dozens of mRNA targets in multiple cell lines, another option would be to send mRNA samples for m6A sequencing. Rather than using m6A-seq also known as MeRip-Seq that is limited by being unable to fully quantify m6A, we would use m6A-SAC-seq [16-18]. M6A-SAC-seq uses an allylic-SAM to label m6A sites to a6m6A followed by cyclization via Iodine (I2) treatment. This protocol is more selective than m6A-seq and attenuates the issue of background noise found when performing m6A-seq [18]. The data attained from m6A-SAC-seq would be consolidated with the sequencing data from our previous studies in order to determine if the changes in mRNA expression were directly caused by a change in m6A of these messages.

Rather than claiming the change is directly related to an m6A site change, we would further characterize this change by determining what mode of regulation the m6A site induced in order for that change to occur. For example, if a target EMT mRNA transcript showed a decrease in mRNA expression with the knockdown of METTL3 as well as a m6A site that is no longer methylated with our m6A-SAC-seq data we would follow up with a polysome profiling assay in order to determine if that m6A site is responsible for a change in translation efficiency. If a different EMT mRNA target or potentially the same target in a different cell line showed an increase in mRNA expression in our sequencing data along with an unmodified m6A site between the negative CRISPR and METTL3 knockdown lines, we would follow up with a mRNA stability assay. This assay would involve using Actinomycin D to halt transcription while largely unaffected replication and translation and measuring the remaining the mRNA at different time points to determine if the transcripts had degraded at a faster rate due to the present m6A modification in the Neg CRISPR lines and if that effect was absent in the METTL3 knockdown cell line with the m6A modification absent. By showing that there are direct differences in m6A sites on EMT transcripts as well

as changes in translation efficiency or mRNA stability, a clear and through link between m6A and EMT can be presented to the field in order to further characterize m6A's dynamic role in breast cancer progression.

Though m6A is a modification found in mRNA, it would be important to determine its effects on protein expression as well. Depending on the selected scope of this future direction, we could potentially run western blots on specific targets that showed change either in our sequencing data presented in this thesis or data that coincides with the results of the previous future direction discussed in order to determine the downstream effects of the m6A modification on the selected EMT target. In our study, we observed that Vimentin protein expression results directly coincided with our migration data in both the MCF10A and MCF10AT1 cell line. With the knockdown of METTL3 in the MCF10A cell line, migration had decreased Vimentin protein expression as well. We observed the opposite effect in the MCF10AT1 cell line in that with the knockdown of METTL3, there was an increase in both migration and Vimentin protein expression. There could potentially be other EMT targets that could directly link m6A with our phenotypical results. If a larger scale analysis is elected to be performed, Mass Spectrometry proteomic analysis can be run on protein samples extracted from our cell lines in order to merge with our transcriptomic analysis to determine the nature of the relationship between mRNA expression and protein expression within our cell lines with the decrease in m6A via the knockdown of METTL3.

Another future direction for us to proceed with is to determine if we see opposite effects in our parental cell lines with the overexpression of METTL3 and METTL14. METTL14 should be overexpressed as well due to METTL3's secondary role in interacting with eif3H in order to circularize mRNAs to enhance translation [19]. By overexpression METTL14, our hypothesis is that we can circumvent METTL3's secondary role due to its direct interaction with METTL14 via both proteins' methyl-transferase domain [20]. One advantage of this future direction is that once the overexpression cell lines are developed, the foundation built by the data presented in this thesis allows for streamlining of the assays and data

analysis. Another advantage is that the future directions presented in this thesis can be run in parallel or subsequently with our knockdown lines.

The data I have presented in this thesis presents a novel viewpoint in m6A's dynamic role in breast cancer progression yet leaves many opportunities to further characterize that dynamic role through the future directions stated for our lab as well as the field as a whole.

## **Clinical Relevance**

Applying the data and its interpretations from the benchtop to bedside is a monumental yet necessary task for researchers. The challenge of utilizing this information in order to produce a treatment for disease that is within a therapeutic window that is a constant moving and shrinking target is difficult enough when the field is in general agreement of the proposed understanding of the observed data. It has been observed that RNA methylation is implicated in not just cancer but viral replication and obesity as well [43,44]. This thesis is focused on m6A in its role in breast cancer, but the ideas represented can be applied to other diseases as well. Without fully characterizing and understanding m6A's dynamic and unique role in cancer based on cell type and stage as well as the vehicle of addressing m6A whether that be attenuating or augmenting m6A levels globally or on specific targets, it would be premature and inefficient to begin looking for potential treatments of disease via m6A itself as well as its writers, erasers, and readers. As mentioned before, depending on several factors, potential treatments may address one or more aspects of disease while exasperating other issues of that same disease. This is especially true in diseases like cancer that can have several subpopulations within a single tumor or tumors found in satellite sites due to metastasis. Furthermore, the several layers of complexity are exponentially more challenging when taking into account the diversity of cancer between patients. In order to efficiently move into treatments of cancer via m6A, further characterization and understanding must be accomplished.

Unfortunately, another challenge at the moment is that treatments affect methylation globally both in non m6A-RNA as well as DNA and Protein. Other treatments inhibit METTL3 such as STC-15 and STM2457 as well as meclofenamic acid and IOX1 that inhibit FTO and ALKBH5 respectively [30-33]. As mentioned before these treatments can have many off target effects due to the nature of the proteins they interact with along with the over encompassing nature of m6A regulation. If a highly specific treatment that has limited off target effects that can still be within the therapeutic window it is unlikely that it alone can cure cancer. Many of the cancer treatment drugs are now being developed in a cocktail of drugs to address the high adaptability of cancer.

## **Conclusion**

In this thesis I have examined the controversy in the literature in regard to m6A's role in both promoting and suppressing several aspects of cancer via its writers, erasers and readers. It also presented novel data that shows m6A's role is dynamic as well as unique based on the stage of cancer as well as the hallmark of cancer being tested as well presented a direct link between m6A and EMT in our MCF10 breast cancer model. It presented data that m6A can augment and attenuate proliferation and migration depending on the stage of breast cancer as well as not having an effect on both phenotypes in late-stage breast cancer. This data was further confirmed in a larger scale transcriptomic analysis of gene clusters that varied with the knockdown of METTL3 and eventual decrease of m6A levels in mRNA that EMT was the most affected biological process in both early-stage breast cancer as well as mid stage breast cancer while also showing that late-stage breast cancer utilized m6A for different biological processes such as interferon response. Lastly it presented mRNA and protein expression data that coincided with phenotypical data in order to begin to characterize a molecular mechanism of the m6A's dynamic and unique role in breast cancer progression and EMT. It then discussed the future directions of the research of this lab group as

well as looking forward in how to address the controversy as well as the gap in knowledge and what are the clinical implications of this research and the overall goal to continue characterizing m6A and its role in cancer progression Overall, I hope this thesis begins a paradigm shift in understanding that m6A is a complex and dynamic regulatory process that can be utilized in a diverse number of ways by cancer cells depending on cell type, stage, m6A player as well as microenvironment both within the tumor and around it. The topic of m6A and cancer has emerged as a major field of study and further understanding, and characterization of this relationship will allow for progress in the eventual goal of utilizing the knowledge gained to producing clinical treatments of cancer.



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