THE ROLE OF N^6-METHYLADENOSINE IN HYPOXIA AND CELLULAR TRANSFORMATION

By

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November, 2017

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Cancer is a prevalent disease that affects millions of people each year across the globe. In an effort to find therapies to the many types of cancers, regulation of protein expression through transcription and translation pathways have been extensively studied. However, one area which has been often overlooked is the importance of post-transcriptional regulation in protein output. The goal of this dissertation project has been to understand post-transcriptional regulation of mRNA during a cellular stress that all cancers must overcome in order to survive, oxygen deprivation. The lack of oxygen, termed hypoxia, is known to affect tumor growth and angiogenesis. Specifically, hypoxia affects the post-transcriptional regulation of mRNAs by increasing the stability of a subset of ischemia-related mRNAs, including VEGF. Multiple factors including RNA binding proteins and miRNAs have been identified to be important for the post-transcriptional regulation of individual mRNAs, but mechanisms regulating global stability have not been elucidated. Recently, the mRNA modification, N^6 methyladenosine (m6A), has been shown to be involved in the post-transcriptional regulation processes of mRNA stability and promotion of translation. Therefore, I set out

to investigate the effect of hypoxia on RNA m6A content. My results show that hypoxic exposure leads to striking changes in the m6A content of mRNA in HEK-293T cells as well as immortalized and oncogenically transformed human mammary epithelial cells (HMECs). Using m6A mRNA immunoprecipitation, we identified a number of specific hypoxia related mRNAs, including Glut1 and c-Myc, which show increased m6A levels under hypoxic conditions. Many of these same mRNAs also exhibit increased mRNA stability revealed by metabolic labeling of RNA using 4sU. Furthermore, knockdown of the m6A-specific methyltransferases METTL3/14 blocked the hypoxic stabilization of these mRNA. The increase in mRNA stability through m6A led to greater translational efficiency after recovery from the hypoxic stress. Overexpressing m6A in oncogenically transformed HMEC in normal oxygen conditions led to an increase in would healing, proliferation, and invasion abilities. Ultimately, the mRNA modification, m6A, led to phenotypic changes in a cancer cell, and it may be possible to manipulate this mRNA modification in order to slow cancer growth.

THE ROLE OF N^6-METHYLADENOSINE IN HYPOXIA AND CELLULAR TRANSFORMATION

A Dissertation

Presented to the Faculty of the Department of Biochemistry and Molecular Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Biochemistry and Molecular Biology

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Dedication

To my amazing wife for support and encouragement through all of my pursuits.

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CHAPTER 1

Introduction: RNA modifications are important for cellular function

N^6-methyladenosine

Deoxyribonucleic acid (DNA) was discovered over 65 years ago as the molecule which stores genetic information within the cell. However, even though DNA is the storage unit for genetic material, the information needs to be translated into a usable form. To make this information functional, the DNA first needs to be transcribed into messenger ribonucleic acid (mRNA) and ultimately translated into protein. Regulation of these transcriptional, post-transcriptional, and translational steps plays a part in determining the functional protein output of the genetic material. Recently, it has been discovered that ribonucleic acid (RNA) modifications play an important role in post-transcriptional and translational regulation. Many modifications, including N^6-methyladenosine (m6A), are found in all types of RNAs [1]. While the functions of a few of these modifications are known, the purpose of many other modifications have yet to be determined. However, early data suggests that many of these modifications are important in cell functionality and could play a role in diseases such as cancer.

There are over 100 known RNA modifications, and it is well established that ribosomal RNA (rRNA) and transfer RNA (tRNA) are rife with modifications. In fact, the most prevalent RNA modification, pseudouridine, is found mostly in rRNA and tRNA [2]. It is for this reason that modifications in these types of RNAs have been well studied. Many of these studied modifications in tRNA and rRNA have been revealed to lead to fine-tuning of their functions in translational efficiency [3]. Interestingly, to date, eukaryotic mRNA has been found to contain at least 13 modifications, including m6A, 5-methylcytidine (m5C), and 7-methylguanosine (m7G) [1], but the function of many of these modifications has yet to be elucidated.

The m6A modification, found in most eukaryotes, is the most abundant modification in mRNA [4], and as such has been the focus of this project. In estimation, there are approximately three m6As per mRNA [5]. This modification has been shown to be important for the stability and translational efficiency of mRNA [6-11], and is involved in the pluripotency of stem cells in embryonic development [12-14] as well as the induction of a cancer stem cell phenotype in breast cancer cells [15, 16]. However, the m6A modification may function in other responses where rapid adaptation to fluctuating cellular environments is required.

mRNA Methyltransferases

The methyltransferase complex required for the formation of the m6A modification on mRNA consists of Methyltransferase like -3 and -14 (METTL3 and METTL14), as well as Wilms' tumor associating protein (WTAP), and is responsible for methylating nascent pre-mRNA within the nucleus [17-21] (Figure 1.1). A couple of reports also suggest that other proteins, including KIAA1429, RBM15, and RBM15B are also a part of the methyltransferase complex as loss of these proteins decrease cellular m6A levels [22, 23]. However, unlike the other components of the methyltransferase complex, the involvement of these proteins in m6A has not been verified by authors outside of the original publications. METTL3 contains an S-adenosyl methionine (SAM) binding domain, and utilizes SAM as a substrate to methylate target adenosines on the nitrogen at the 6^{th} carbon residue on mRNA that contain a DRACH m6A consensus sequence, often found in 3` UTR's and around both start and stop codons [17, 24-27]. METTL14 lacks catalytic activity but participates in mRNA binding/targeting [28-30]. WTAP is responsible for the localization of the Mettl3/14 complex to the nuclear

Figure 1.1

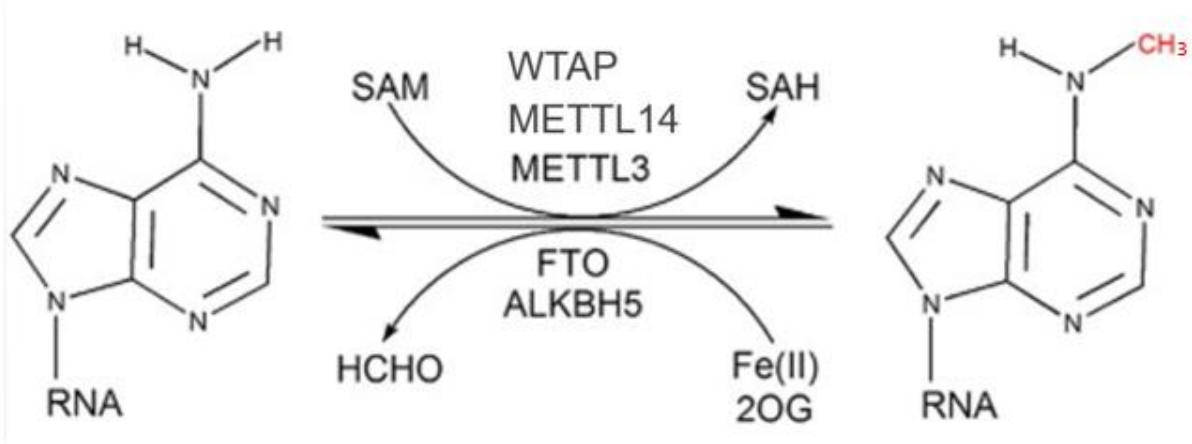
SAM
WTAP
METTL14
METTL3
SAH
FTO
ALKBH5
HCHO
Fe(II)
2OG
RNA
RNA
CH3

Figure 1.1: m6A methylation reaction

An adenosine is methylated at the N6 position replacing a hydrogen. The methyl group is shown in red on the second half of the figure. The methyltransferase complex consisting of METTL3, METTL14, and WTAP mediates this methylation. METTL3 contains the SAM binding domain which catalyzes the donation of a methyl group from SAM and releasing S-adenosyl homocysteine (SAH). M6A methylation is reversible and demethylation occurs through FTO and ALKBH5 which are Fe^{II} and α-ketoglutarate (2OG) dependent and releasing formaldehyde (HCHO) as a byproduct [31-33].

speckle, and greatly enhances methyltransferase activity by bringing the methyltransferase to the pre-mRNA. However, WTAP is not ultimately required for methyltransferase activity [18, 34]. Although predominantly found in the nucleus, the methyltransferase complex, along with methyltransferase activity has been found within the cytoplasm [18, 35-38]. Interestingly, a recent study has shown that m6A methylation is not dynamic within the cytoplasm [21]. This study showed that the methylated adenosines in the nucleus were still methylated in the cytoplasm with no additionally methylated adenosines. This data suggests that m6A methylation is not dynamic in the cytoplasm.

Demethylases

m6A methylation of RNA is reversible and can be reportedly removed by alkylation repair homolog 5 (ALKBH5) and fat mass and obesity related protein (FTO) [39-48]. Together, these enzymes make up 2 of the 9 ALKB family members which are Fe^{II} and α-ketoglutarate (α-KG) dependent [31, 32] (Figure 1.1).

Previously, FTO has been of major interest to the obesity field after it was identified that a single nucleotide polymorphism (SNP) in intron 1 of the FTO gene is associated with increased BMI and adiposity through genome wide association studies (GWAS) [49]. However, the link between FTO and obesity has recently been suggested to be indirect. An SNP in intron 1 of the FTO gene leads to the overexpression of two nearby genes, IRX3 and IRX5. The overexpression of these two genes then leads to a decrease in mitochondrial energy production and an increase in lipid accumulation [50, 51]. Therefore, the function of FTO is no longer definitively linked to obesity.

Even though ALKBH5 and FTO enzymes are closely related, the mechanism of m6A demethylation differ between the two. m6A demethylation through FTO yields two intermediate nucleotides, N^6 -hydroxymethyladenosine (hm6A) and N^6 -formyladenosine (fm6A), while demethylation through ALKBH5 yields no observable intermediates [44, 52]. Because of this difference, it is possible that RNA binding proteins (RBPs) may interact with these intermediate RNA modifications during FTO mediated demethylation. Therefore, it may be important to determine which enzyme is active in the demethylation process in each model. A recent study has shown that FTO does not preferentially bind m6A, but rather N^6,2′-*O*-dimethyladenosine (m6Am) [53]. In addition, previous reports showed that m6A sites were unaffected in FTO-deficient mice, and it is known that the antibodies used in early m6A mapping studies can bind m6Am as well [54, 55]. This new data suggests that FTO is not an m6A demethylase, but rather an m6Am demethylase. However, the many previous reports describing FTO as an m6A demethylase cannot all be discounted.

RNA Binding Proteins

m6A methylated mRNA is bound by RBPs that bind directly to the site of modification, including many members of the YTH family [9, 56, 57]. m6A also affects other RBPs that do not interact directly with the m6A site, but instead are affected by the RNA secondary structure caused by the modification [58, 59]. Interestingly, m6A affects double stranded RNA by rotating the face of the adenosine from the more energetically favorable “syn” confirmation to its less favorable “anti” confirmation [60]. This confirmation switching destabilizes the RNA duplex. However, m6A sites in single strand RNA leads to higher stability through stronger stacking ability with its nucleotide

neighbors. Generally speaking, stretches of RNA containing the m6A modification tends to prefer a single stranded structure, however the overall structure of the RNA will be dependent on many m6A sites as well as other factors of RNA structure [60].

The m6A modification has been shown to affect the binding of other RBPs including heterogeneous nuclear ribonucleoprotein (HNRNP)G and HNRNPC; in fact, over 13,000 m6A sites regulate RNA-HNRNPG interactions [58, 59]. In summary, the m6A modification not only directly interact with RBPs, but also indirectly regulate other RBPs through modifying RNA secondary structure which may lead to many downstream effects including changes in RNA stability and enhanced translational efficiency.

While much is known about the mechanisms of m6A writing and erasing, the broader consequences of RNA methylation are still being investigated. It appears likely that the m6A binding proteins will ultimately determine the consequences of RNA methylation. This project has revealed that m6A can lead to stability of mRNA in hypoxic conditions [11]. However, in previous studies, m6A methylation has been shown to mark mRNA for degradation, mediated by YTHDF2 transport to P bodies where degradation of mRNA occurs through DCP1/DCP2 decapping and CCR4-NOT deadenylation [6, 9, 23, 40, 61]. Additionally, YTHDF1 has been reported to interact with initiation factors as well as enhance ribosomal loading in order to stimulate translational efficiency of m6A methylated mRNA thereby increasing translational output [10]. YTHDF3 and YTHDC2 have also been shown to promote translation of m6A methylated mRNA [62, 63], and it has been recently discovered that YTHDC2 is required for mouse spermatogenesis [63]. Another YTH family member that binds m6A, YTHDC1, interacts with splicing factors in order to regulate RNA splicing [57, 64]. (Table 1.1)

Table 1.1

m6A RNA Binding Protein	Function	Reference(s)
YTHDF1	Interact with initiation factors and enhance ribosomal loading to stimulate translational efficiency	[10]
YTHDF2	Degradation of mRNA after transport of m6A mRNA to P-bodies	[6, 9, 23, 40, 61]
YTHDF3	Promotes Translation	[62]
YTHDC1	Regulates mRNA splicing	[57, 64]
YTHDC2	Promotes Translation	[63]

The interaction or competition between the YTH family of proteins (and other m6A binding proteins) is not yet understood as it has yet to be determined why binding of one takes precedence over another. However, we do know that YTHDF1, 2, 3, and YTHDC2 are cytoplasmic, whereas YTHDC1 is nuclear [9, 56, 63, 64]. It may be possible that YTH binding is tissue or cell type specific, or, alternatively, YTH binding is competitive. It is possible that certain cell conditions or signaling will favor the binding of one YTH family member over another. Certainly, there is much more to understand about m6A binding proteins to fully grasp the overall consequences of RNA methylation.

m6A in non-coding RNAs

Even though m6A is prevalent in mRNA, it is also found in other types of RNAs including rRNA, long non-coding RNA (lncRNA), microRNA, and small nuclear RNA (snRNA) [65-68]. The m6A modification in microRNA has been reported to aid in pri-microRNA processing through the RBP DGCR8 [69, 70]. In fact, the RBP HNRNPA2B1 which interacts with DGCR8 is a nuclear reader of m6A. Loss of HNRNPA2B1 or Mettl3 led to similar losses in microRNA production [70].

The m6A modification is also found in snRNA's including U6 snRNA. Interestingly, a completely different Mettl family protein, Mettl16, is responsible for the m6A methylation of U6 snRNA [71]. It seems likely that Mettl16 is a methyltransferase for many other RNA targets, and in fact unpublished data from our lab suggest that Mettl16 methylates 18S rRNA as well as a handful of mRNAs.

Surprisingly, the m6A modification is only found in one location on both the 18S and 28S rRNA species at base 1832 and 4180 respectively, but has not been detected

on 5 or 5.8S RNAs [72]. Interestingly, even though most rRNA modifications are highly conserved in eukaryotes, the m6A modification has not been detected in yeast rRNA [73]. Although the function of these modifications are not yet known, structure predictions informs us that the m6A sites are located in important areas for translational fidelity. The m6A site in the 18S rRNA is located at the top of helix 44 in the mRNA binding pocket, and the 28S m6A is near the 5S rRNA interaction site [74, 75] (Figure 1.2) [73]. These structure predictions, along with the fact that other rRNA modifications have been reported to be required for rRNA formation and function, suggests that the m6A modification may play an important role in translation [76, 77].

Function

Understanding of the RBP's that are regulated by or directly interact with m6A may lead us to better grasp the functional aspect of m6A. Currently, one of the biggest questions and challenges in the m6A field is correlating changes in m6A RNA with functional outputs. The RNA modification field is attempting to answer these questions, and many strides are being made. For example, the highly methylated long-noncoding RNA, *XIST*, along with m6A RBP YTHDC1 has been indicated to be involved in gene transcription silencing on the X chromosome [22]. In fact, loss of m6A on *XIST* impairs its function of silencing gene transcription on the X chromosome, but artificial binding of YTHDC1 to the m6A depleted *XIST* rescues its activity. Additionally, m6A has been implicated in the ultraviolet DNA damage response [34]. m6A methylated RNA briefly accumulates at DNA damage sites after UV irradiation, and in the absence of Mettl3, the DNA damage response was greatly slowed [34].

Figure 1.2

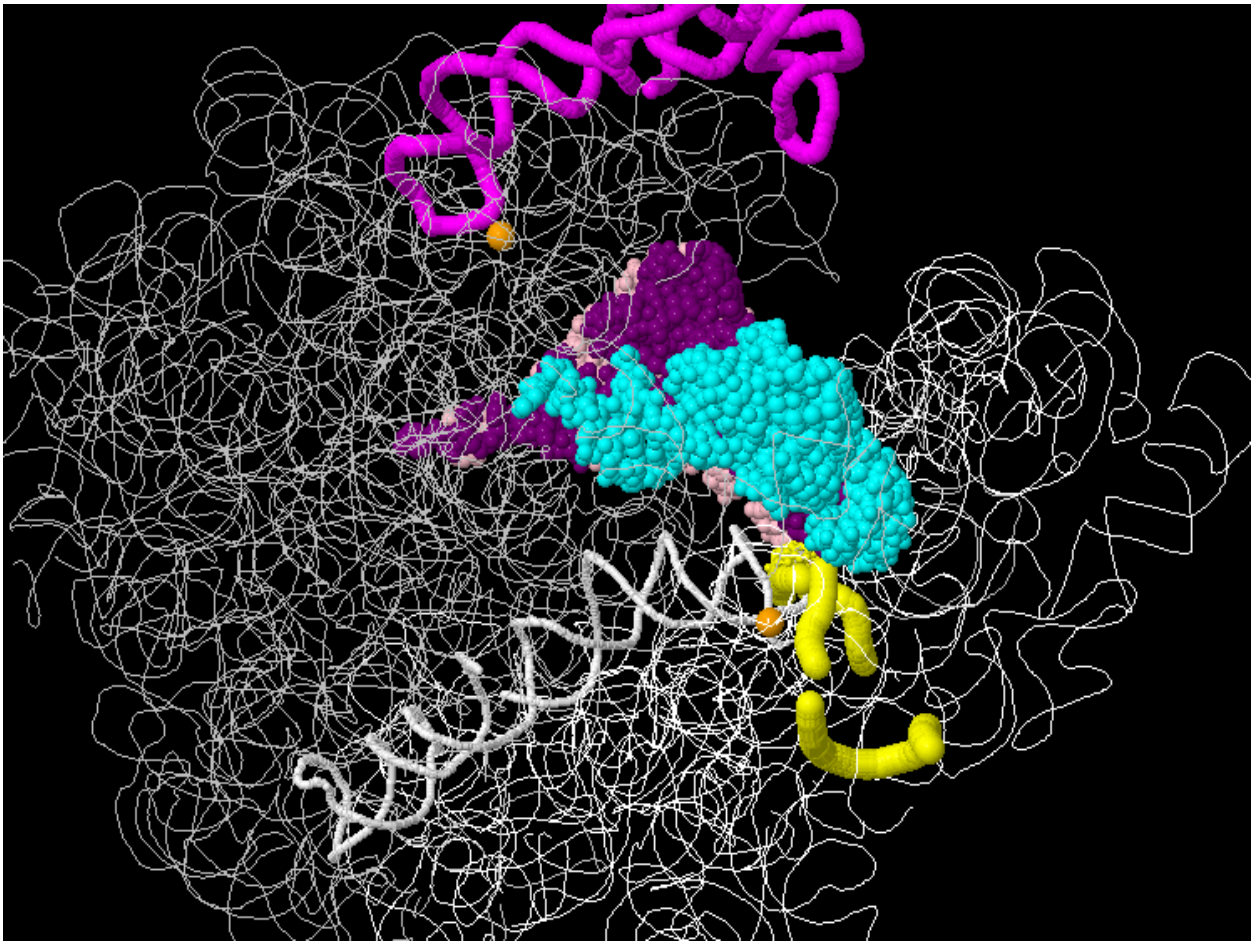

Figure 1.2: Ribosomal rRNA with m6A modifications

Predictive structure of small and large rRNA subunits together. Orange balls indicate m6A sites. White Helix is helix 44. Yellow ribbon is mRNA in the mRNA binding pocket. Green and purple clusters indicate tRNA binding sites. Purple Ribbon is 5S rRNA.

Interestingly, the m6A modification has also been found on many viral RNA genomes [36, 78-83]. It is well known that viruses evolve rapidly to evade environmental pressure, so it is assumed that any trait that is preserved must give an evolutionary advantage. Three different groups published reports that m6A in the viral RNA genome of HIV-1 increased infectivity of the virus through enhanced mRNA expression [79, 80, 83]. This result could be attenuated by depleting YTHDF1,2, and 3 proteins which ultimately inhibited HIV-1 replication. This data suggests that YTHDF proteins may be potential targets of therapies of HIV-1 infected individuals. Inversely, however, it has been reported that m6A in a *Flaviviridae* family members, hepatitis C virus (HCV) and Zika virus, decreases infectivity [36, 81].

m6A has also been reported to be important in embryonic stem cell differentiation [12, 13, 84]. A number of reports have suggested that m6A methylation is needed to drive embryonic stem cells towards differentiation. Loss of the methyltransferase complex leads to increased self-renewal and decreased differentiation of the stem cells [12, 84, 85] These reports have suggested that m6A specifically regulates expression of pluripotency genes including Nanog and Sox2 as well as signaling genes Notch1 and RhoCA [84, 85]. In contrast, an additional report suggested that m6A methylation drives stem cell self-renewal rather than differentiation [13]. This contrasting reported showed that developmental regulators including Sox1 and IgfBP3 were highly methylated and destabilized whereas pluripotency genes like Sox2 exhibited lower methylation and increased stability leading to self-renewal. One reviewer has suggested that the difference in these reports may be that m6A levels do not regulate the transition of stem cells to differentiated cells, but rather fine tune these events through RNA stability [14].

This review suggests that stem cells exist in two states, a ground naïve state and a primed state ready to differentiate, and m6A simply aids in driving the continuation of the current state of the cell.

Not only has m6A been revealed to regulate embryonic stem cells, but cancer stem cells as well, including breast cancer and glioblastoma stem cells [15, 86]. In both models, loss of m6A leads to enhanced cancer stem cell self-renewal and growth. Increased ALKBH5, leading to decreased m6A levels, in glioblastoma stem cells has also been directly implicated in increased tumorigenicity and self-renewal [87]. Exposure of breast cancer stem cells to a hypoxic environment leads to increased expression of the pluripotency factor NANOG through the reduction of m6A [15]. m6A levels were reduced in the breast cancer stem cells through an increase in the demethylase ALKBH5, as well sequestration of Mettl3 via increased levels of ZNF217 [16, 88]. ZNF217 has previously been reported to be a poor prognosis marker in breast cancer glioma [89, 90]. In an additional model, decreased m6A in hepatocellular carcinoma (HCC) through the downregulation of Mettl14 also led to increased metastasis in HCC and poor patient prognosis [91]. Inversely, overexpression of Mettl14, leading to increased m6A, decreased metastasis in HCC. However, this same study showed that increased Mettl3 also increased the invasive ability of HCC. Another group also found that Mettl3 promotes translation in lung adenocarcinoma cells leading to increased growth and invasion of human lung cancer cells [38].

As of yet, it has been difficult to reconcile these two differing results. One group shows that as part of the methyltransferase complex Mettl14 decreases, thereby decreasing m6A, cancer migration and invasion increase. However, the second study

shows that the other part of the methyltransferase complex, Mettl3, which increases m6A, promotes growth and invasion of cancer. Three possible explanations may account for this discrepancy. The first being that these two types of cancers are different, and therefore, the effect of m6A will not be the same on both. Another possible explanation is through the independent functions of Mettl3 and Mettl14. In the lung adenocarcinoma model, Mettl3 increased translation in the cancer cells [38]. However in the HCC model, Mettl14 regulated splicing of miR126 which has been identified as a metastasis suppressor [91]. So in the absence of Mettl14, mature miR126 was not available to suppress metastasis. In these cases, it may be possible that m6A levels are merely a side effect from the increased or decreased methyltransferase carrying out alternative functions. A third option may be that a decrease in Mettl14 in the HCC model may actually lead to free Mettl3 independently increasing translation. This possibility may actually account for both sides of the problem as the decreased miR126 and increased translation may both promote increased cell invasion.

Hypoxia

Understanding m6A's role in hypoxia is vital because the hypoxic condition can occur during embryonic development and tumor growth where crowding and rapid cell division causes a shortage of blood and oxygen supply [92, 93]. Because changes in m6A have been implicated in the phenotypes of both embryonic and cancer stem cells, it is important to understand what effect the physiological presence of hypoxia will have on m6A, and ultimately on the cell's phenotype. The amount of oxygen required for cells can vary depending on their location and function. For example, normal oxygen levels for lung cells is 5.6%, but normal oxygen levels in the muscle is around 4% [94].

However, under hypoxic conditions, the cell becomes stressed and must overcome the low oxygen in order to survive. Alternatively, if the cell cannot overcome hypoxia, the cell may undergo apoptosis. Many tumors, however, not only survive in hypoxic conditions, they thrive [95-100].

The hypoxic response is initiated by the stabilization of the hypoxia inducible factor-1α (HIF)-1α protein. HIF-1 is made up of two subunits. Under normal conditions HIF-1β is constitutively active whereas HIF-1α is degraded through binding of the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex. The VHL- HIF-1α complex is mediated by hydroxylation of two proline residues via three members of a Fe(II)- and 2-oxoglutarate–dependent oxygenase family called prolyl hydroxylase (PHD) 1,2, and 3 [101-107]. HIF-1α acts as a transcription factor that binds to genes with a hypoxia response element (HRE) in order to promote their transcription [108-111]. The hypoxic response is utilized in tumor growth because of its ability to turn on cellular pathways which are necessary for tumorigenesis. HIF-1α directly stimulates the transcription of VEGF, thus leading to increased tumor vascularization. HIF-1α also aids tumor cell migration through the upregulation of cell migration genes including C-X-C chemokine receptor type 4 (CXCR4) [96, 99, 112-115] mesenchymal epithelial transition factor (c-MET), and insulin-like growth factor binding protein 1 (IGFBP1) [114, 116, 117].

Recently, it has become clear that hypoxia not only has transcriptional regulation effects, but also affects post-transcriptional regulation. The stability of a subset of mRNA targets, including vascular endothelial growth factor (VEGF) and Glucose Transporter 1 (Glut1), increases during hypoxic stress [118]. For example, the stability of VEGF mRNA increases VEGF protein translation which will lead to an increase in

signaling to VEGFR-2 receptors on endothelial cells ultimately increasing angiogenesis [119] in order to receive a greater blood supply thereby leading more oxygen to the area [120-123].

The Mansfield lab has recently expanded upon these studies and identified numerous mRNAs stabilized in response to oxygen and glucose deprivation including VEGF, MYC, Hairy and Enhancer of Split 1 (Hes1), JUN, and Dual Specificity Protein Phosphatase 1 (DUSP1) [124]. As stated above, m6A plays a clear role in stability of mRNA, and my work has confirmed that m6A is required for the hypoxic stabilization of a subset of targets [11]. Because hypoxia is so important to tumor cell proliferation and migration, it is important to fully understand gene regulation during hypoxia, including regulation through m6A methylation.

This dissertation project aims to advance the field of m6A modification by understanding its role in post-transcriptional regulation of mRNA during hypoxia. m6A has previously been reported to be involved in numerous post-transcriptional regulation processes including mRNA stability, splicing, and translational efficiency. In order to accomplish these regulation tasks, an RBP as described above must lead the methylated mRNA to its fate. Understanding the role of m6A methylation on the post-transcriptional regulation processes in hypoxia will lead to a greater understanding of how post-transcriptional events lead to phenotypic changes in cells.

CHAPTER 2

N6-methyladenosine is required for the hypoxic stabilization of specific mRNAs

The majority of the work presented in this chapter was published as “N6-methyladenosine is required for the hypoxic stabilization of specific mRNAs”

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RNA 23:1444-1455 June 2017

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ABSTRACT

Post-transcriptional regulation of mRNA during oxygen deprivation, or hypoxia, can affect the survivability of cells. Hypoxia has been shown to increase stability of a subset of ischemia-related mRNAs, including VEGF. RNA binding proteins and miRNAs have been identified as important for post-transcriptional regulation of individual mRNAs, but corresponding mechanisms that regulate global stability are not well understood. Recently, mRNA modification by N^6-methyladenosine (m6A) has been shown to be involved in post-transcriptional regulation processes including mRNA stability and promotion of translation, but the role of m6A in the hypoxia response is unknown. In this study, we investigate the effect of hypoxia on RNA modifications including m6A. Our results show hypoxia increases m6A content of $PolyA^+$ messenger RNA (mRNA), but not in total or ribosomal RNA in HEK293T cells. Using m6A mRNA immunoprecipitation, we identify specific hypoxia-modified mRNAs, including Glut1 and c-Myc, which show increased m6A levels under hypoxic conditions. Many of these mRNAs also exhibit increased stability, which was blocked by knockdown of m6A-specific methyltransferases METTL3/14. However, the increase in mRNA stability did not correlate with a change in translational efficiency or the steady-state amount of their proteins. Knockdown of METTL3/14 did reveal that m6A is involved in recovery of translational efficiency after hypoxic stress. Therefore, our results suggest that an increase in m6A mRNA during hypoxic exposure leads to post-transcriptional stabilization of specific mRNAs and contributes to the recovery of translational efficiency after hypoxic stress.

Introduction

Hypoxia is a metabolic condition that occurs when oxygen levels are deficient in cells or tissues. This stress can occur during embryonic development, where crowding and rapid cell division causes a shortage of blood and oxygen supply [92, 93]. Hypoxia can also be brought on by impaired blood flow due to heart attack or stroke [125, 126]. Other diseases can affect oxygen delivery including sickle cell disease and low blood pressure, which create hypoxic environments within the tissues. Regardless of the origins of the hypoxic stress, cells must alter their metabolism and gene expression in ways that will increase their chance of survival, or succumb to apoptosis.

The physiological response to hypoxia is initiated by the stabilization of the HIF-1α transcription factor targeting genes containing a HRE [108-111]. HIF-1α is also important for promoting cancer cell survival through interactions with Myc and Jun, and it is well documented that hypoxia drives tumor angiogenesis [95-99, 127, 128]. HIF-1α directly stimulates the transcription of VEGF. This hallmark of the hypoxic response leads to increased translation of VEGF promoting vascular growth in order to increase the blood supply to affected cells, thereby leading to increased oxygen [120-123]. The hypoxic response also aids tumor migration by upregulating the genes that are involved in the degradation of the extracellular matrix, as well as increasing the metastatic ability of the tumor and cellular proliferation through genes such as Dusp1 and Hes1 [96, 99, 112, 113, 115, 129-131]. Because the hypoxic response is so important to cancer cell survival, it is critical to fully understand all mechanisms occurring during hypoxia including post-transcriptional regulation.

Although the transcriptional response to hypoxia is well established, the post-transcriptional response to oxygen deprivation is less understood. Post-transcriptional responses often regulate mRNA splicing and stability, and the stability of individual mRNAs, such as VEGF and Glut1 is increased with hypoxic exposure [118]. We have recently expanded upon these studies and identified numerous mRNAs stabilized in response to oxygen and glucose deprivation including VEGF, MYC, Hes1, JUN, and DUSP1 [124]. Specific sequences in VEGF mRNA 3' UTR and ORF have also been discovered to contribute to the stabilization of VEGF in response to hypoxia, but this analysis has not been extended to other mRNAs [121, 122, 132-134]. It has also been well documented that severe oxygen deprivation leads to inhibition of global cap-dependent translation, but post-transcriptional regulation of a subset of mRNAs allows for continued translation through a number of proposed mechanisms [135-140]. The hypoxic response has also recently been implicated in global changes in alternative splicing [141, 142]. Thus, it is clear that post-transcriptional regulation of mRNAs has a role in the hypoxic response, but the mechanisms involving this regulation have not all been identified.

Recently, m6A mRNA modification has been shown to be important for the stability and translational efficiency of mRNA [6-10]. m6A methylation is a post-transcriptional modification of mRNA occurring in the nucleus [17, 18, 20]. The m6A methyltransferase complex consists of Methyltransferase like -3 and -14 (METTL3 and METTL14), as well as WTAP [17-19]. METTL3 contains a SAM binding domain, and utilizes SAM as a substrate to methylate target mRNAs that contain a DRACH m6A consensus sequence, often found in 3` UTR's and around stop codons [17, 24], while

METTL14 lacks catalytic activity but participates in mRNA binding/targeting [28-30]. m6A methylation of RNA is reversible and can be removed by ALKBH5 or FTO [39-46]. Methylated mRNA is transported out of the nucleus and bound by RNA binding proteins, including most members of the YTH family [9, 56, 57]. While much is known about the mechanisms of m6A writing and erasing, the broader consequences of RNA methylation are still being investigated. m6A methylation has been shown to mark mRNA for degradation, mediated by YTHDF2 transport to P bodies where degradation of mRNA occurs [9, 23, 40, 61]. Additionally, YTHDF1 has recently been reported to stimulate translational efficiency of m6A methylated mRNA thereby increasing translational output [10].

It is known that through post-transcriptional regulation, the fate of mRNAs can change based on the changing conditions of the cell [143]. Post-transcriptional modifications like m6A may alter the fate of RNA by potentially altering secondary structure, the ability of RNA binding proteins to bind, or the position of splicing events [58, 60, 68, 144, 145]. Changes at the RNA level can occur rapidly, and are necessary to adapt to rapidly changing microenvironments. RNA modifications can also direct permanent changes within the cell, as is the case of m6A modifications that limit the pluripotency of embryonic stem cells [12-14]. Defects in these post-transcriptional modifications could cause problems in rapid cell response mechanisms, embryonic development, or even promote tumor growth.

m6A mRNA has received much attention in recent years, allowing the factors involved in m6A mRNA methylation to be identified. However, the importance of dynamic mRNA methylation has remained elusive. Recently, roles for m6A mRNA in the

pluripotency of stem cells in embryonic development [12-14], as well as the induction of a cancer stem cell phenotype in breast cancer cells has been described [15, 16]. However, the m6A modification may function in other responses, including adaptation to changing cellular environments. Therefore, it is quite possible that the m6A modification may be involved in post-transcriptional hypoxic response mechanisms. Understanding the shifting landscape of m6A mRNA in hypoxic cells will shed light on not only how post-transcriptional regulation is altered when oxygen is lacking, but also the extent of m6A post-transcriptional utilization within a cell under other dynamic conditions.

Results

Effect of Hypoxia on cellular RNA modification levels

Changes in the m6A modification of mRNA have been shown to regulate stem cell pluripotency during embryonic development as well as breast cancer stem cell phenotypes. We investigated whether a cellular stress that is present in both of these models, hypoxia, has any effect on RNA modification levels, including m6A. To do this, HEK293T cells were incubated for 24 hours under normoxic or hypoxic (1% O_2) conditions in the presence of 1 g/L glucose to mimic the nutrient deprivation encountered during ischemic events. Total RNA was isolated, dotted onto a nylon membrane, and m6A levels were detected with an m6A-specific antibody. As shown in Figure 2.1A, exposure of HEK293T cells to hypoxic conditions resulted in a dramatic decrease in the total RNA m6A content as detected by the m6A antibody. To further elucidate which RNA species were being affected by hypoxia, PolyA$^+$ mRNA was

enriched by oligo-dT selection followed by rRNA depletion (Figure 2.2), and after fragmentation, liquid chromatography and tandem mass spectrometry (LC-MS) was used to quantify various RNA modifications in the mRNA enriched samples. Surprisingly, in contrast with a recent report measuring m6A in total RNA and specific mRNAs in breast cancer cells [15], global PolyA$^+$ RNA showed a significant increase in m6A content in HEK293T cells exposed to hypoxia (Figure 2.1E). This result was confirmed by m6A dot blot (Figure 2.1B). Other mRNA modifications, including 5-methylcytidine, showed no significant differences in hypoxia (Figure 2.3). This data shows that the mRNA modification, m6A, has a dynamic response to hypoxia.

As rRNA makes up 80-85% of the cellular RNA mass, we postulated that diminished M6A content in total RNA observed here and by others [15] derived chiefly from changes in rRNA methylation. This was demonstrated directly by isolating 40 and 60S ribosomal subunits via differential centrifugation through sucrose gradients. rRNA was isolated from the fractions, subjected to qPCR to verify the composition of the fractions (Figure 2.4), and then dot blotted for M6A. A clear decrease in m6A content in both 18S and 28S rRNA in HEK293T cells exposed to hypoxia was shown (Figure 2.1C). We used LC-MS to confirm these changes in total, and rRNA, and contrary to our dot blot results, m6A levels were unchanged in hypoxia (Figure 2.1 D, F). However, 5-methylcytidine and N1-methylguanosine were significantly decreased in total RNA (Figure 2.5). Similarly to total RNA, 5-methylcytidine exhibited a statistically significant decrease in content in 18S RNA, and N1-methylguanosine exhibited a decreasing trend in 18S RNA as well (Figure 2.6). Interestingly, Pseudouridine also exhibited a decrease

Figure 2.1

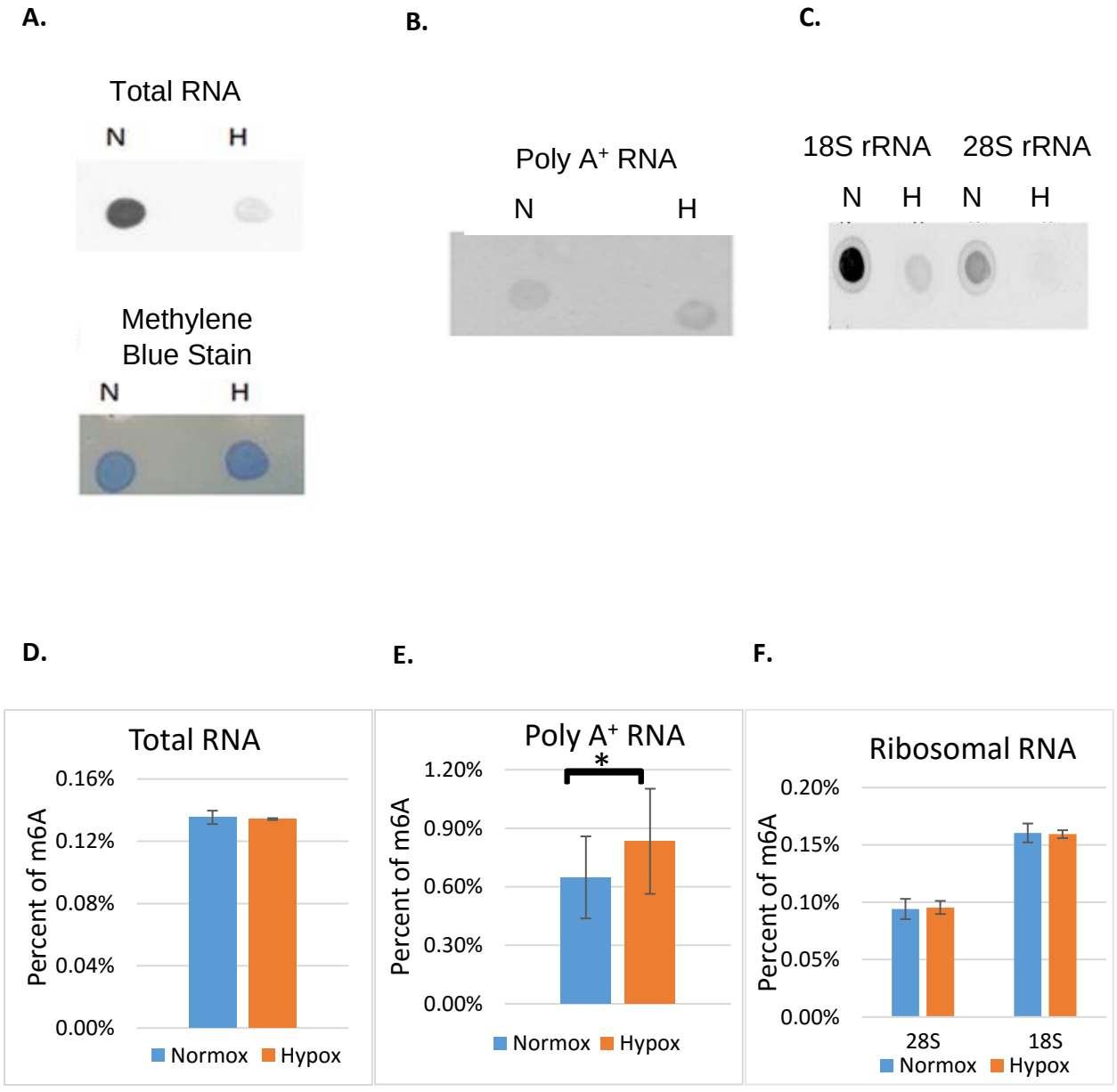

A.
Total RNA
N
H
Methylene Blue Stain
N
H
B.
Poly A⁺ RNA
N
H
C.
18S rRNA
28S rRNA
N
H
N
H
D.
Total RNA
Percent of m6A
0.16%
0.12%
0.08%
0.04%
0.00%
Normox
Hypox
E.
Poly A⁺ RNA
*
Percent of m6A
1.20%
0.90%
0.60%
0.30%
0.00%
Normox
Hypox
F.
Ribosomal RNA
Percent of m6A
0.20%
0.15%
0.10%
0.05%
0.00%
28S
18S
Normox
Hypox

Figure 2.1: m6A levels in total, Poly A$^+$, and rRNA

(A) RNA isolated from HEK-293T cells grown in Normoxic (N) or Hypoxic (H) conditions for 24 hours. Top Panel. Immunoblot with m6A antibody of 1 µg of total RNA. Bottom Panel. Methylene Blue Stain of total RNA showing equal loading of RNA. (Image is representative of 5 biological replicates). (B) m6A Immunoblot of 100 ng of PolyA RNA from HEK-293T cells grown in Normoxic or Hypoxic conditions. (Image is representative of 3 biological replicates). (C) m6A Immunoblot of 1 µg 18S and 28S ribosomal rRNA isolated via differential centrifugation through sucrose gradients. (Image is representative of 3 biological replicates). (D) LC-MS/MS of Total RNA from HEK-293T cells. Values represent the amount of m6A divided by total Adenosine (N of 2). (E) LC-MS/MS of mRNA from HEK-293T cells. Values represent the amount of m6A divided by total Adenosine (N of 5). (F) LC-MS/MS of 18 and 28S rRNA from HEK-293T cells. Values represent the amount of m6A divided by total Adenosine (N of 3).

*P ≤ 0.05 by Paired Student's t-test. Error bars represent standard error of the mean (SEM).

Figure 2.2

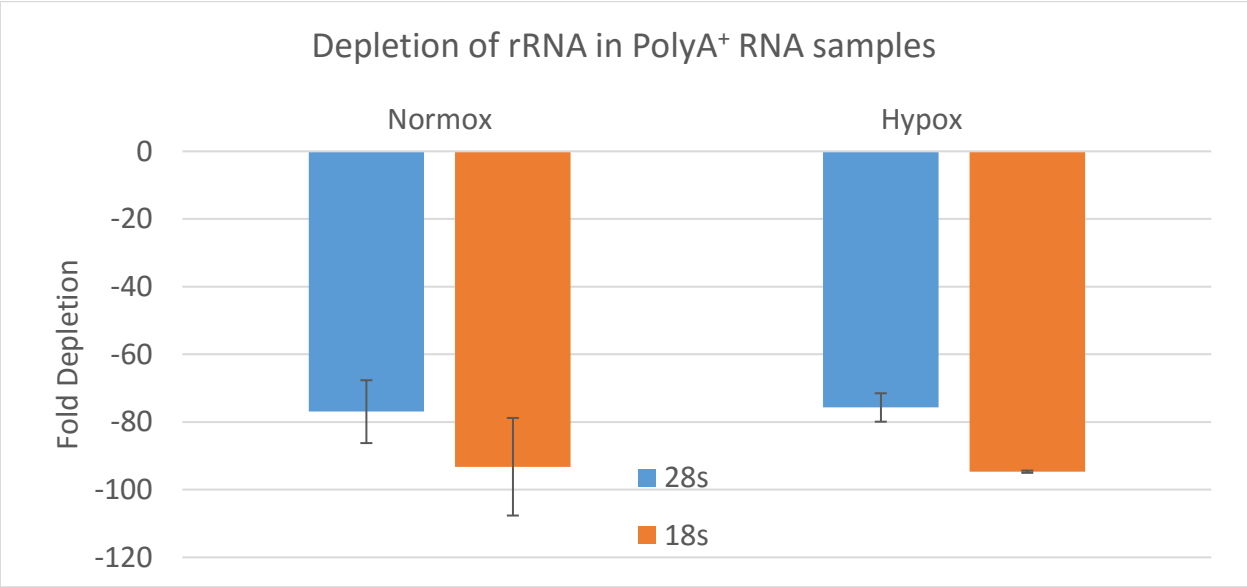

Depletion of rRNA in PolyA+ RNA samples
Normox
Hypox
0
-20
-40
-60
-80
-100
-120
Fold Depletion
28s
18s

Figure 2.2: Depletion of rRNA in PolyA$^+$ RNA samples

qRT-PCR verification of rRNA depletion in PolyA$^+$ RNA. Both 18 and 28S rRNA from cells cultured in Normoxic or 24 hours Hypoxic conditions were depleted over 70-fold indicating greater than 98% depletion of rRNA. (N of 2)

Figure 2.3

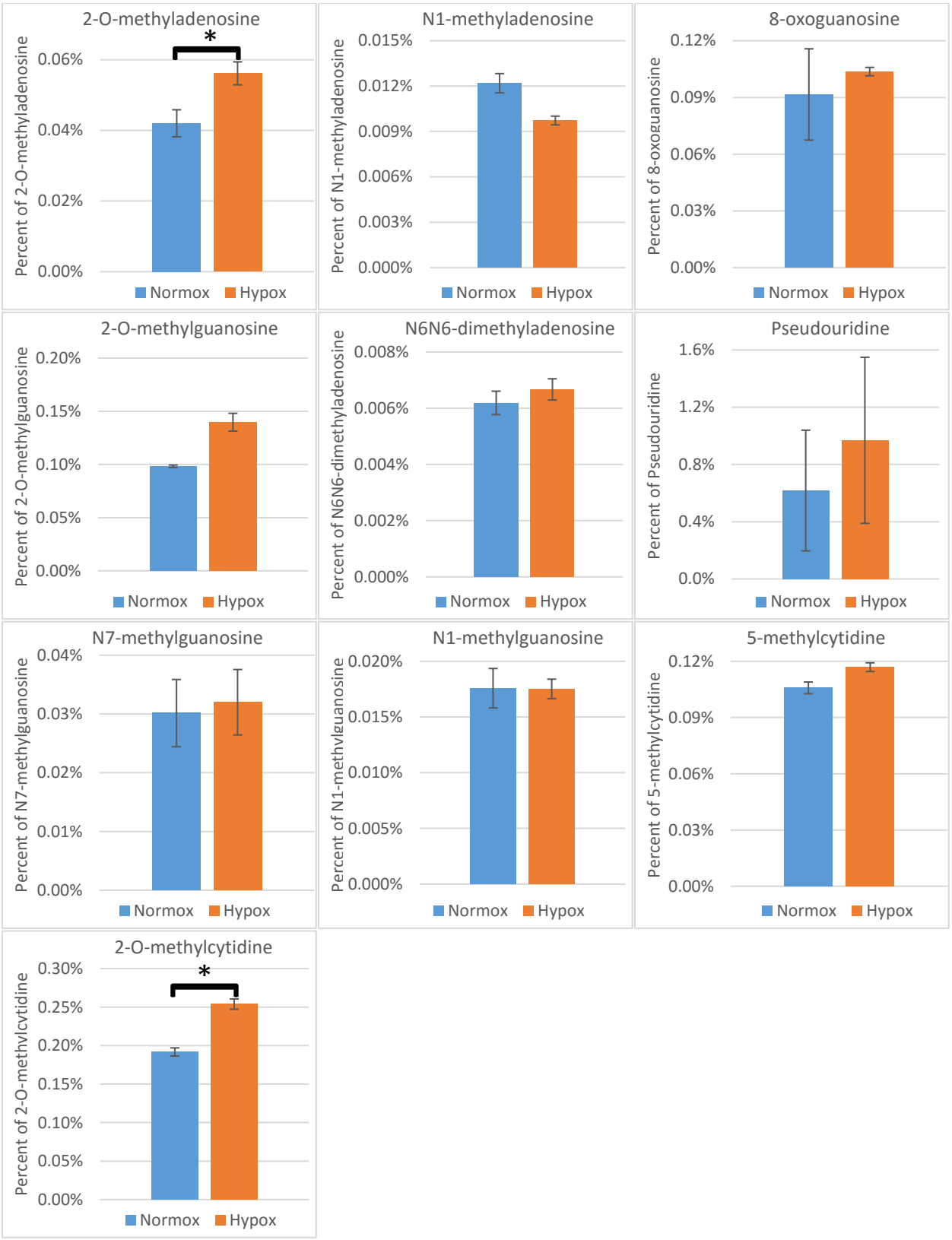

2-O-methyladenosine
Percent of 2-O-methyladenosine
0.06%
0.04%
0.02%
0.00%
*
Normox
Hypox
N1-methyladenosine
Percent of N1-methyladenosine
0.015%
0.012%
0.009%
0.006%
0.003%
0.000%
Normox
Hypox
8-oxoguanosine
Percent of 8-oxoguanosine
0.12%
0.09%
0.06%
0.03%
0.00%
Normox
Hypox
2-O-methylguanosine
Percent of 2-O-methylguanosine
0.20%
0.15%
0.10%
0.05%
0.00%
Normox
Hypox
N6N6-dimethyladenosine
Percent of N6N6-dimethyladenosine
0.008%
0.006%
0.004%
0.002%
0.000%
Normox
Hypox
Pseudouridine
Percent of Pseudouridine
1.6%
1.2%
0.8%
0.4%
0.0%
Normox
Hypox
N7-methylguanosine
Percent of N7-methylguanosine
0.04%
0.03%
0.02%
0.01%
0.00%
Normox
Hypox
N1-methylguanosine
Percent of N1-methylguanosine
0.020%
0.015%
0.010%
0.005%
0.000%
Normox
Hypox
5-methylcytidine
Percent of 5-methylcytidine
0.12%
0.09%
0.06%
0.03%
0.00%
Normox
Hypox
2-O-methylcytidine
Percent of 2-O-methylcytidine
0.30%
0.25%
0.20%
0.15%
0.10%
0.05%
0.00%
*
Normox
Hypox

Figure 2.3 UPLC-MS/MS of Poly A$^+$ RNA from HEK-293T cells. Values represent the amount of the modification divided by total parent levels (N of 2). *P ≤ 0.05 by Paired Student's t-test. Error bars represent Standard error of the mean (SEM).

Figure 2.4

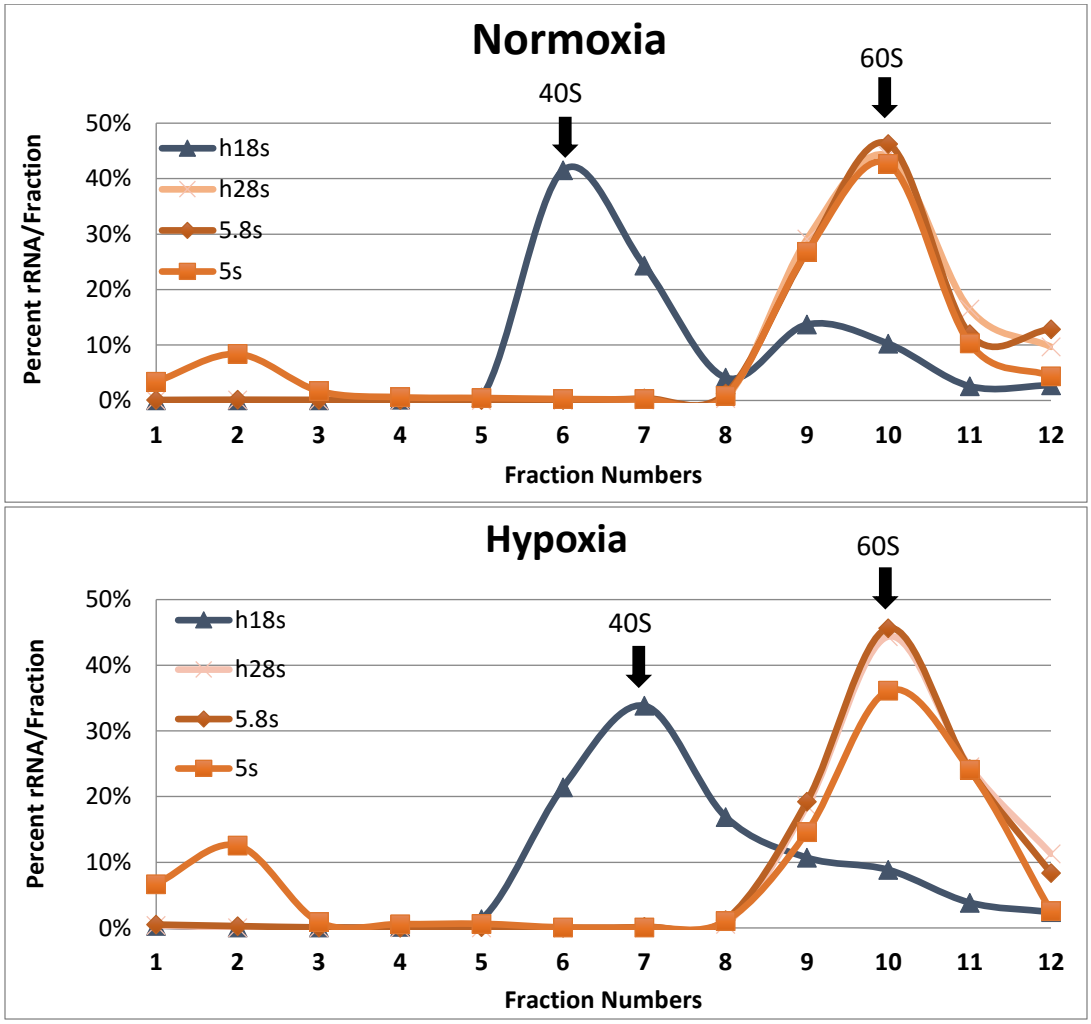

Normoxia
60S
40S
50%
40%
30%
20%
10%
0%
h18s
h28s
5.8s
5s
Percent rRNA/Fraction
1 2 3 4 5 6 7 8 9 10 11 12
Fraction Numbers
Hypoxia
60S
40S
50%
40%
30%
20%
10%
0%
h18s
h28s
5.8s
5s
Percent rRNA/Fraction
1 2 3 4 5 6 7 8 9 10 11 12
Fraction Numbers

Figure 2.4: Confirmation of ribosomal sub-units

40 and 60S ribosomal subunits from HEK-293T cells grown in normoxic or hypoxic conditions for 24 hours were separated by differential centrifugation followed by sucrose gradient fractionation. qRT-PCR of individual fractions reveals purity of respective rRNAs in each fraction. Fractions containing 40S and 60S peaks are marked. Fractions with highest purity were used in immunoblot in Figure 2.1C. (Graph represents 1 of 3 experiments).

in content in the isolated 28S rRNA, but no changes were detected in total RNA (Figure 2.5 and 2.6). These results suggest that not only mRNA modifications, but also specific rRNA modifications are dynamically altered after 24 hours of hypoxic conditions. However, for this study we chose to focus on the effects of hypoxia on the mRNA m6A content given its reported effects on mRNA regulation.

Identification of differentially methylated mRNAs

Given that we saw dynamic changes in the m6A content of mRNA, we next wanted to determine if m6A methylation of specific mRNA targets involved in the adaptive response was affected when cells were exposed to hypoxia. Using m6A RNA immunoprecipitation (MeRIP), target mRNAs related to hypoxia and tumorigenesis including Glut1, Jun, Myc, DUSP1, and Hes1 were investigated. HEK293T cells were exposed to 24 hours of normoxia or hypoxia and PolyA mRNA isolated via oligo dt selection and ribosomal RNA depletion. m6A containing mRNAs were then immunoprecipitated using an m6A-specific antibody. Following cDNA synthesis, the relative enrichment of the indicated mRNAs were determined using qPCR. The targets were quantified relative to input RNA and the negative IP. Interestingly, many of the hypoxia-associated and tumorigenic mRNAs from hypoxic cells, including Glut1 and Jun, increased more than 2-fold in the m6A captured fraction, as compared to the normoxic conditions (Figure 2.7). This signifies an increase in the m6A content of these mRNAs in response to hypoxic exposure. It is likely that these and other mRNAs contribute to the enhanced m6A content of PolyA$^+$ RNA (Figure 2.2E). Importantly, mRNAs such as Human antigen R (HuR) and METTL3 showed no change in m6A content (Figure 2.7), suggesting that this was not a global phenomenon, but rather a

Figure 2.5

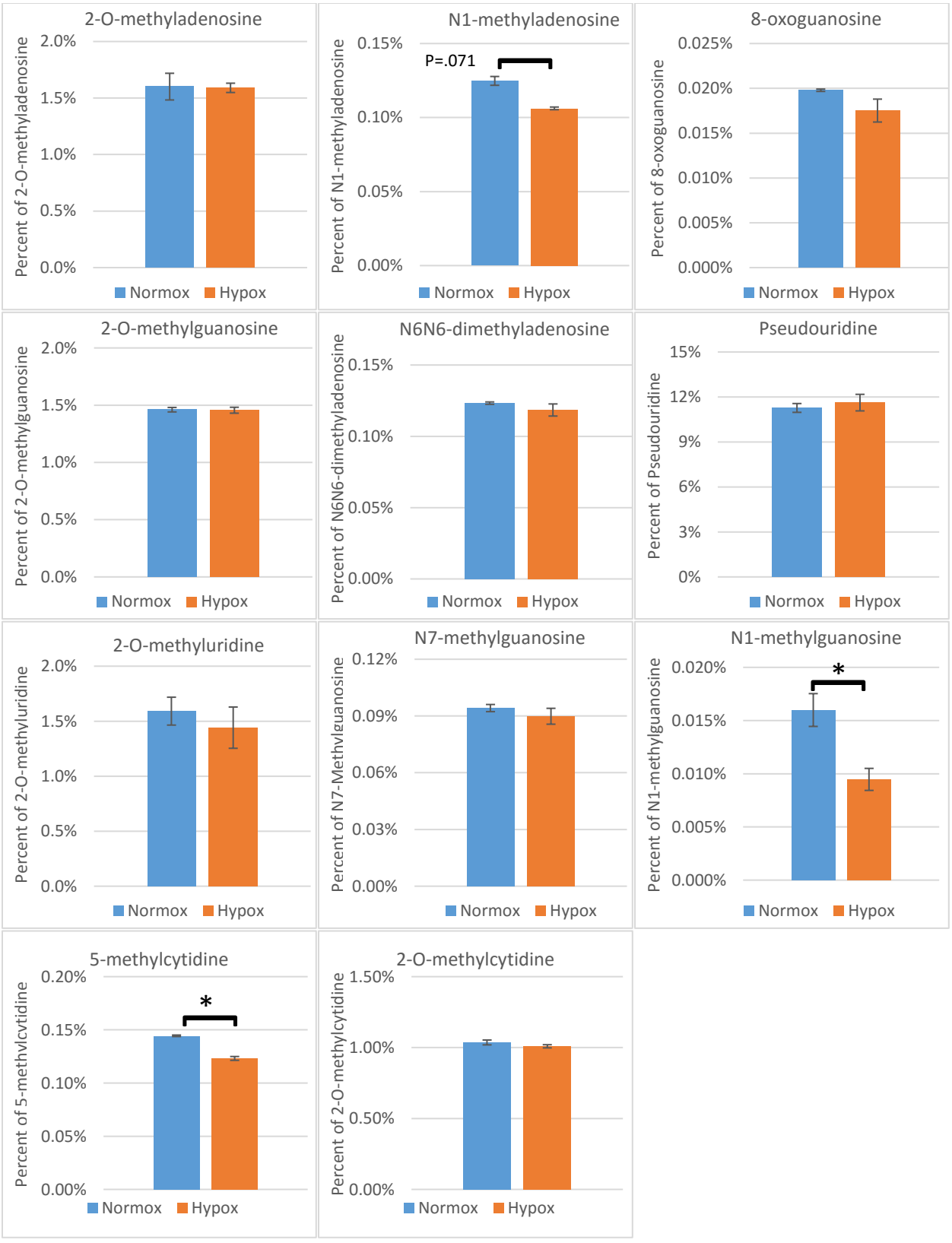

2-O-methyladenosine
Percent of 2-O-methyladenosine
2.0%
1.5%
1.0%
0.5%
0.0%
Normox
Hypox
N1-methyladenosine
Percent of N1-methyladenosine
0.15%
0.10%
0.05%
0.00%
P=.071
Normox
Hypox
8-oxoguanosine
Percent of 8-oxoguanosine
0.025%
0.020%
0.015%
0.010%
0.005%
0.000%
Normox
Hypox
2-O-methylguanosine
Percent of 2-O-methylguanosine
2.0%
1.5%
1.0%
0.5%
0.0%
Normox
Hypox
N6N6-dimethyladenosine
Percent of N6N6-dimethyladenosine
0.15%
0.10%
0.05%
0.00%
Normox
Hypox
Pseudouridine
Percent of Pseudouridine
15%
12%
9%
6%
3%
0%
Normox
Hypox
2-O-methyluridine
Percent of 2-O-methyluridine
2.0%
1.5%
1.0%
0.5%
0.0%
Normox
Hypox
N7-methylguanosine
Percent of N7-Methylguanosine
0.12%
0.09%
0.06%
0.03%
0.00%
Normox
Hypox
N1-methylguanosine
Percent of N1-methylguanosine
0.020%
0.015%
0.010%
0.005%
0.000%
*
Normox
Hypox
5-methylcytidine
Percent of 5-methylcytidine
0.20%
0.15%
0.10%
0.05%
0.00%
*
Normox
Hypox
2-O-methylcytidine
Percent of 2-O-methylcytidine
1.50%
1.00%
0.50%
0.00%
Normox
Hypox

Figure 2.5. UPLC-MS/MS of Total RNA from HEK-293T cells. Values represent the amount of the modification divided by total parent levels (N of 2). *P ≤ 0.05 by Paired Student's t-test. Error bars represent Standard error of the mean (SEM).

Figure 2.6

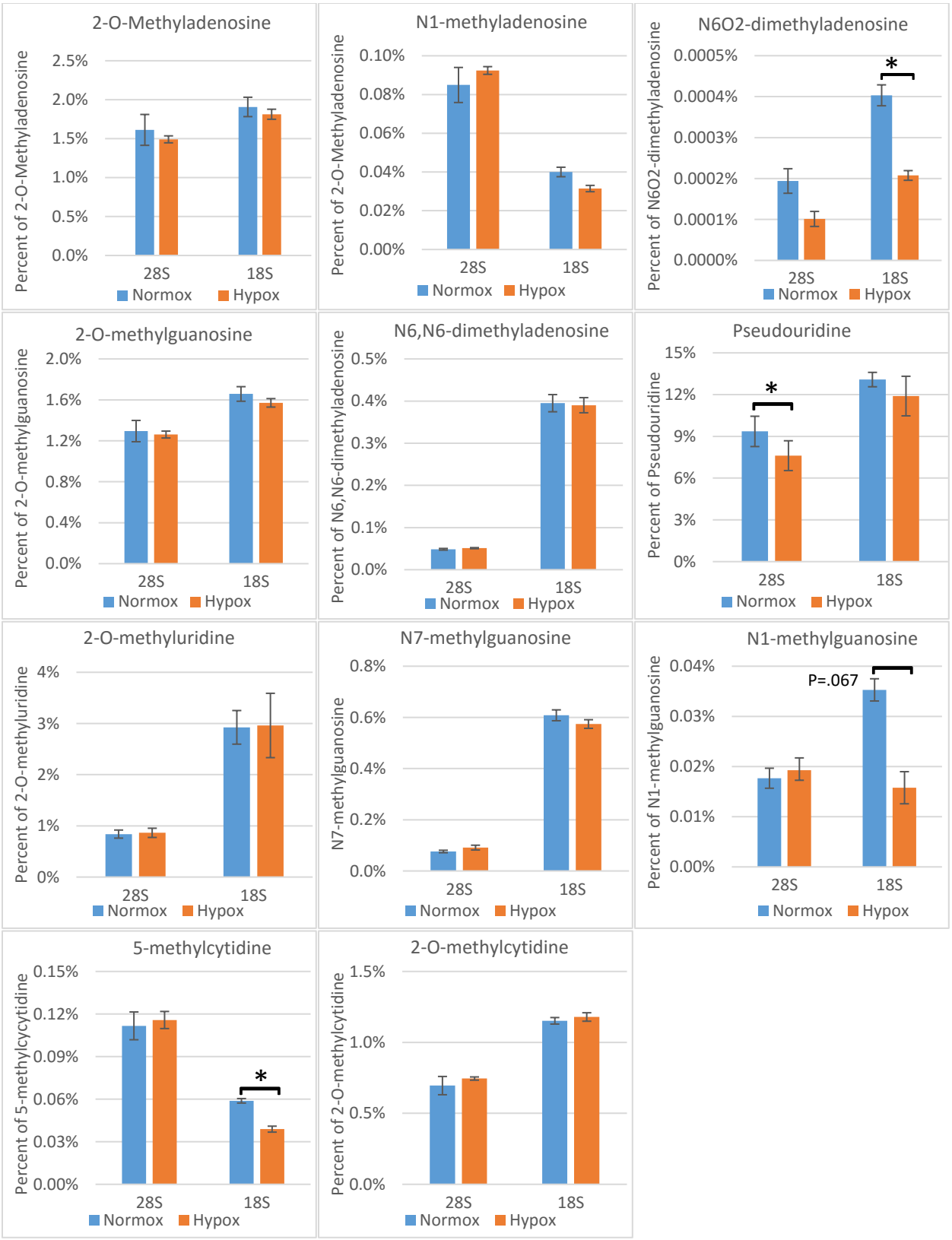

2-O-Methyladenosine
Percent of 2-O-Methyladenosine
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
28S
18S
Normox
Hypox
N1-methyladenosine
Percent of 2-O-Methyladenosine
0.10%
0.08%
0.06%
0.04%
0.02%
0.00%
28S
18S
Normox
Hypox
N6O2-dimethyladenosine
Percent of N6O2-dimethyladenosine
0.0005%
0.0004%
0.0003%
0.0002%
0.0001%
0.0000%
*
28S
18S
Normox
Hypox
2-O-methylguanosine
Percent of 2-O-methylguanosine
2.0%
1.6%
1.2%
0.8%
0.4%
0.0%
28S
18S
Normox
Hypox
N6,N6-dimethyladenosine
Percent of N6,N6-dimethyladenosine
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
28S
18S
Normox
Hypox
Pseudouridine
Percent of Pseudouridine
15%
12%
9%
6%
3%
0%
*
28S
18S
Normox
Hypox
2-O-methyluridine
Percent of 2-O-methyluridine
4%
3%
2%
1%
0%
28S
18S
Normox
Hypox
N7-methylguanosine
N7-methylguanosine
0.8%
0.6%
0.4%
0.2%
0.0%
28S
18S
Normox
Hypox
N1-methylguanosine
Percent of N1-methylguanosine
0.04%
0.03%
0.02%
0.01%
0.00%
P=.067
28S
18S
Normox
Hypox
5-methylcytidine
Percent of 5-methylcytidine
0.15%
0.12%
0.09%
0.06%
0.03%
0.00%
*
28S
18S
Normox
Hypox
2-O-methylcytidine
Percent of 2-O-methylcytidine
1.5%
1.0%
0.5%
0.0%
28S
18S
Normox
Hypox

Figure 2.6. UPLC-MS/MS of Ribosomal RNA from HEK-293T cells. Values represent the amount of the modification divided by total parent levels (N of 3). *P ≤ 0.05 by Paired Student's t-test. Error bars represent Standard error of the mean (SEM).

Figure 2.7

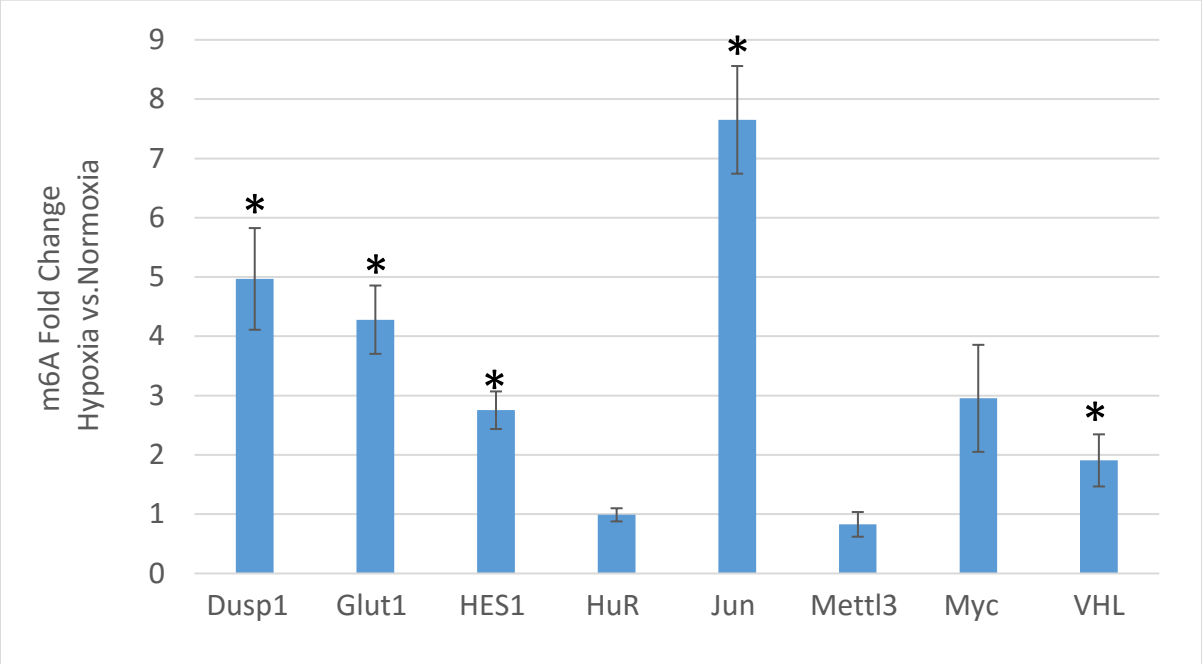

m6A Fold Change
Hypoxia vs.Normoxia
9
8
7
6
5
4
3
2
1
0
*
*
*
*
*
Dusp1
Glut1
HES1
HuR
Jun
Mettl3
Myc
VHL

Figure 2.7: m6A increases in individual targets after hypoxia

MeRIP of 100 ng of mRNA from HEK-293T cells grown in Normoxic or Hypoxic conditions for 24 hours quantified by qRT-PCR. Fold enrichments calculated from immunoprecipitated mRNA levels normalized to input and bead-only negative control IP and expressed as a ratio of hypoxia/normoxia. *P ≤ 0.05 by Paired Student's t-test relative to normoxic m6A levels. Error bars represent SEM of 5 experiments.

directed change.

Loss of m6A Prevents Hypoxic mRNA stabilization

To address if m6A methylation plays a role in stabilization of these particular mRNAs as part of their post-transcriptional response to hypoxia, METTL3 and METTL14 of the m6A methyltransferase complex were knocked down via siRNA and the half-lives of our target mRNAs were determined. Knockdown of METTL3 and METTL14 was confirmed via western blot analysis (Figure 2.8). Depletion of m6A content in individual mRNAs was confirmed by MeRIP followed by qPCR (Figure 2.9A). After depletion of METTL3 and METTL14 for 48 hours, HEK293T cells were transferred to either normoxic or hypoxic conditions for 24 hours to simulate ischemia. During the last hour of treatment, newly transcribed RNA was metabolically labeled using 4-thiouridine (4sU). RNA was harvested, the 4sU labeled RNA was biotinylated, and streptavidin beads were used to separate the new labeled RNA from unlabeled RNA. RNA half-lives were determined from the ratio of the labeled to unlabeled RNA normalized to total RNA. As shown in Figure 2.9B, many of the mRNAs that had increased m6A methylation in hypoxic conditions (Glut1, Jun, Dusp1 and HES1) also showed a significant increase in mRNA half-life. Interestingly, knockdown of METTL3/14 had no effect on the normoxic half-life of any of the mRNAs, but loss of the methyltransferases did significantly inhibit their hypoxic stabilization. eEFA1, which exhibited no change in m6A level (data not shown) also showed no loss of stabilization after METTL3/14 depletion. Therefore, mRNAs that were stabilized under hypoxic conditions lost that stabilization after an engineered decrease in m6A levels. This suggests that enhanced m6A modification of specific mRNAs can indeed affect their

Figure 2.8

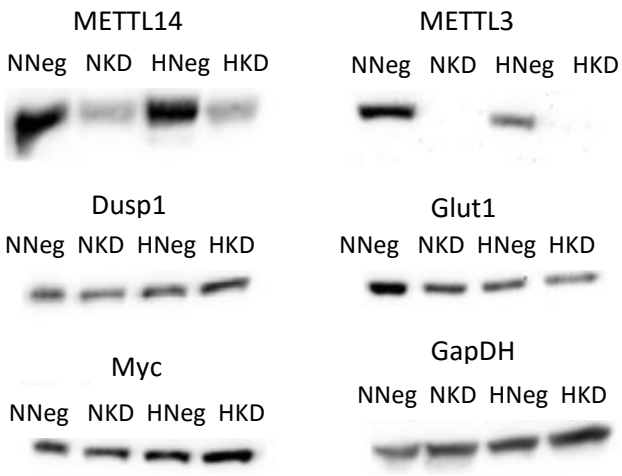
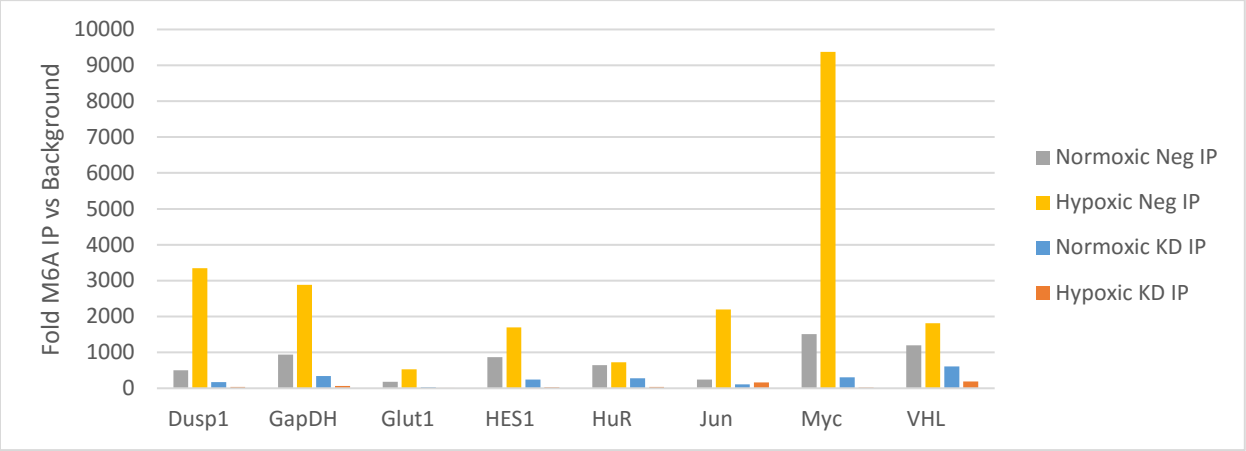

METTL14
NNeg NKD HNeg HKD
METTL3
NNeg NKD HNeg HKD
Dusp1
NNeg NKD HNeg HKD
Glut1
NNeg NKD HNeg HKD
Myc
NNeg NKD HNeg HKD
GapDH
NNeg NKD HNeg HKD

Figure 2.8: Mettl3 and 14 KD confirmation and Protein output is not affected by loss of m6A. HEK-293T cells harvested after 72 hours transfection with METTLl3/14 (M3/14) or negative control (Neg) siRNA and 24 hours of normoxic or hypoxic conditions. Western blots of 50 µg of protein lysates. (Image is representative of 3 biological replicates)

Figure 2.9

A.


Fold M6A IP vs Background
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Dusp1
GapDH
Glut1
HES1
HuR
Jun
Myc
VHL
Normoxic Neg IP
Hypoxic Neg IP
Normoxic KD IP
Hypoxic KD IP

B.

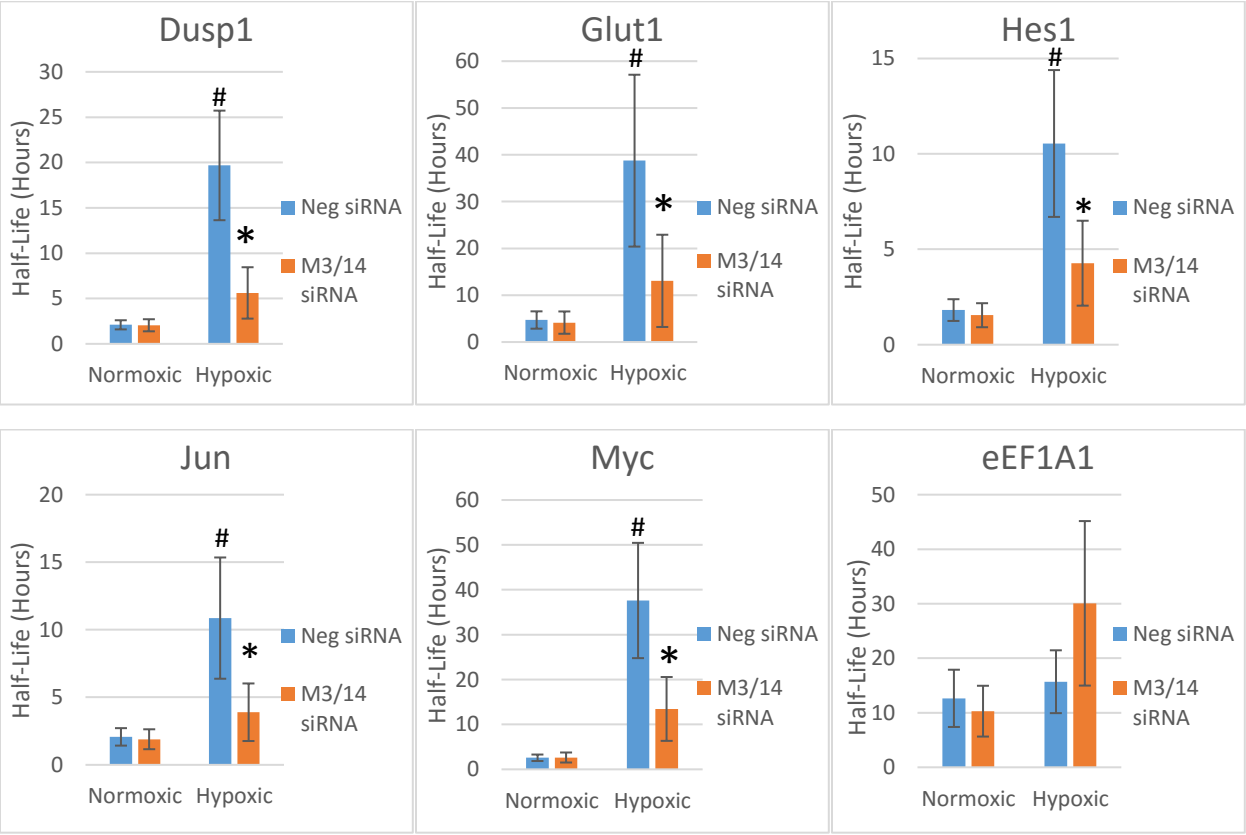

Dusp1
Glut1
Hes1
Jun
Myc
eEF1A1
Half-Life (Hours)
Normoxic
Hypoxic
Neg siRNA
M3/14 siRNA
#
*

Figure 2.9: Transfection with METTL3 and METTL14 siRNAs decrease m6A levels. (A). HEK-293Ts transfected with METTL3 and 14 siRNAs (KD) or negative control siRNA (Neg) for 72 hours and 24 hours of normoxic or hypoxic exposure followed by MeRIP and qRT-PCR quantitation shows decreased M6A levels in mRNA targets after Mettl3/14 knockdown. (N of 1) (B)Total RNA from HEK-293T cells was harvested after 72 hours transfection with METTL3/14 (M3/14) or negative control (Neg) siRNA and 24 hours of normoxic or hypoxic conditions and half-life determined via 4SU. # denotes P ≤ 0.05 by Paired Student's t-test between negative siRNA samples in normoxic and hypoxic conditions while *denotes P ≤ 0.05 by Paired Student's t-test between negative and knockdown siRNAs in the hypoxic condition. Error bars represent SEM of 5 experiments.

hypoxic post-trasncriptional regulation.

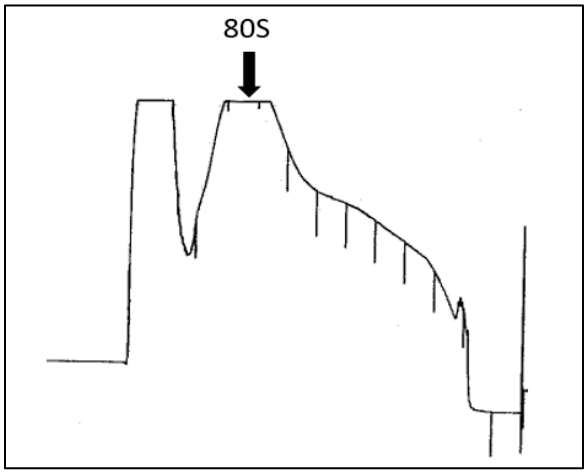
The effect of m6A on mRNA translation and protein levels

Having determined that changes in m6A content affected mRNA stabilization, we next examined how methylation might affect the translation of these messages. HEK293T cells were again depleted of METTL3/14 for 48 hours, and transferred to either normoxic or hypoxic conditions for 24 hours followed by 15 minutes of cyclohexamide treatment and fractionation of cellular extracts on sucrose gradients. Polysome profiles (Figure 2.10 A-D) were analyzed by qRT-PCR for specific mRNA targets. Sedimentation position as shown by polysome profiling allowed us to determine how efficiently each message was being translated. During sucrose gradient resolution, mRNAs partition based on the number of ribosomes bound, which is a direct measure of their translational efficiency. For example, mRNA such as Beta-2-Microglobulin (β2M) which is found to peak in fraction 8 in normoxic conditions, is considered to be moderately translated, while an mRNA that is primarily located in fractions 10 and 11, such as Glut1, is bound heavily by polysomes indicating highly efficient translation (Figure 2.11). In general, hypoxic exposure caused a decrease in translational efficiency (as indicated by a left-ward shift in the gradient) of assayed mRNAs. The extent of the decrease depended on the mRNA being investigated. Surprisingly, depletion of m6A via METTL3/14 knockdown had no effect on the polysome loading of any of the tested mRNAs, suggesting that m6A does not play a role in their translational efficiency (Figure 2.11).

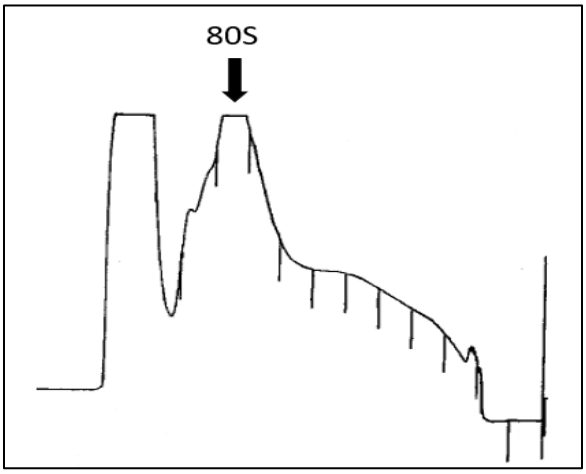
To determine if increased m6A during hypoxia was involved in the recovery after hypoxia, polysome profiling was used to determine translational efficiency after

Figure 2.10

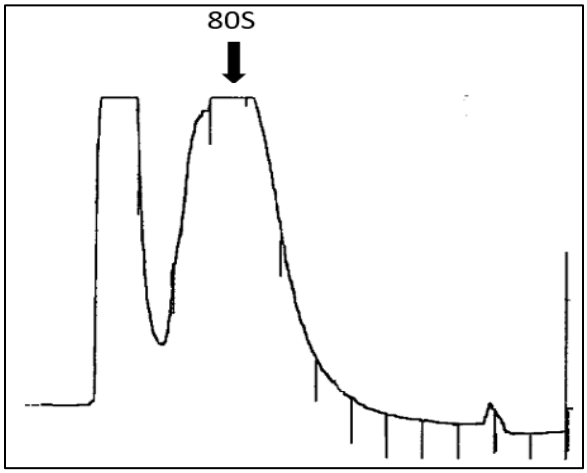
A.



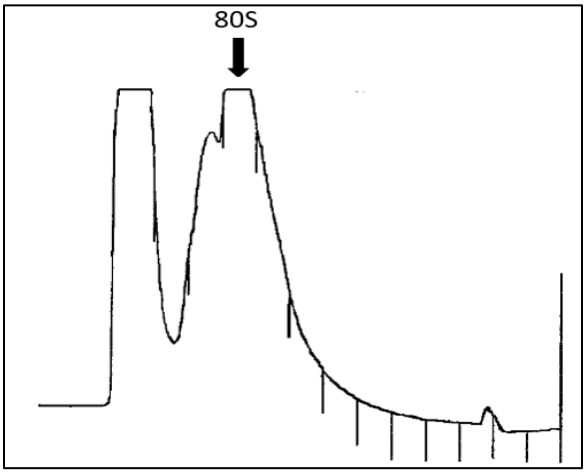
B.



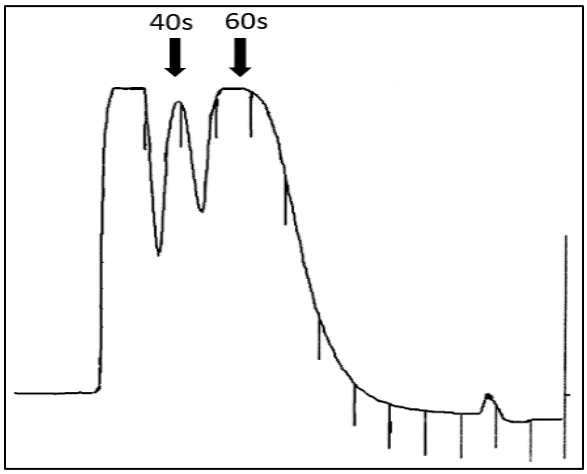
C.



D.



E.



F.

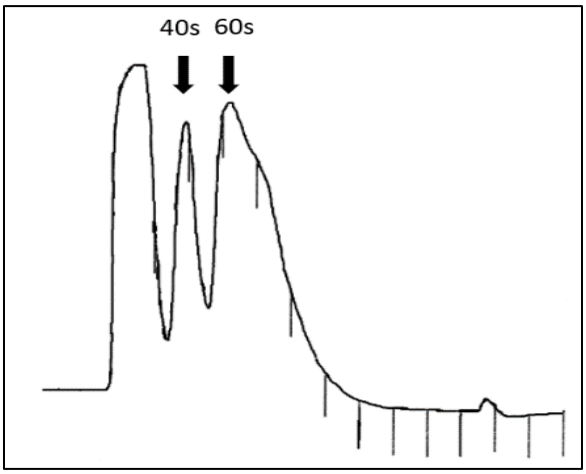


Figure 2.10: Polysome Profile traces.

(A-D) Traces of Polysome Profiles from Figure 2.11. (A), Normoxia, (B), Normoxia M3/14 siRNA, (C), Hypoxia, (D) Hypoxia M3/14 siRNA. (E-F) Traces of Polysome Profiles from Figure 18. (E) Normoxia, (F), Hypoxia. (Traces represent 1 of 3 experiments).

Figure 2.11

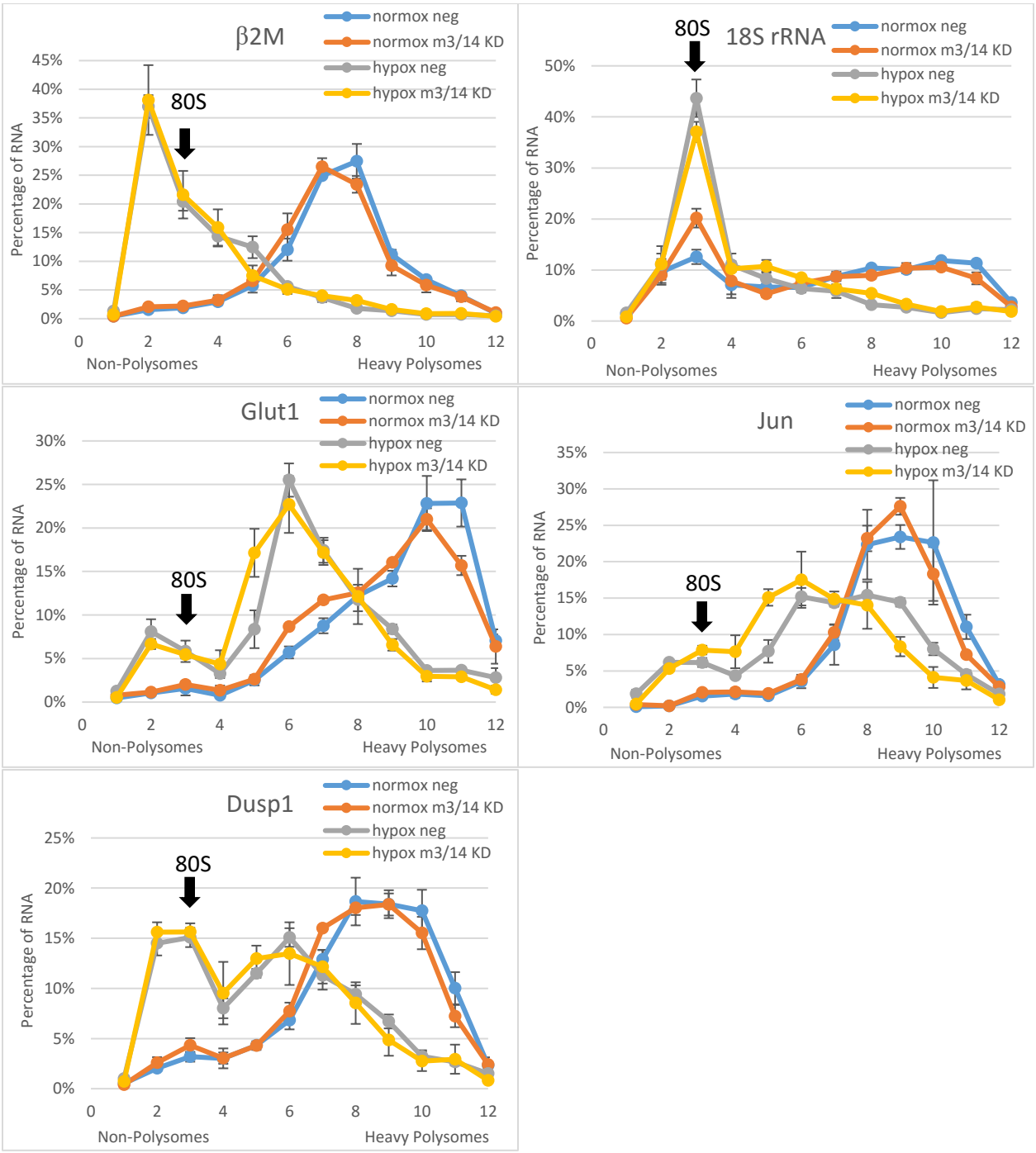

β2M
normox neg
normox m3/14 KD
hypox neg
hypox m3/14 KD
80S
Percentage of RNA
Non-Polysomes
Heavy Polysomes
18S rRNA
Glut1
Jun
Dusp1

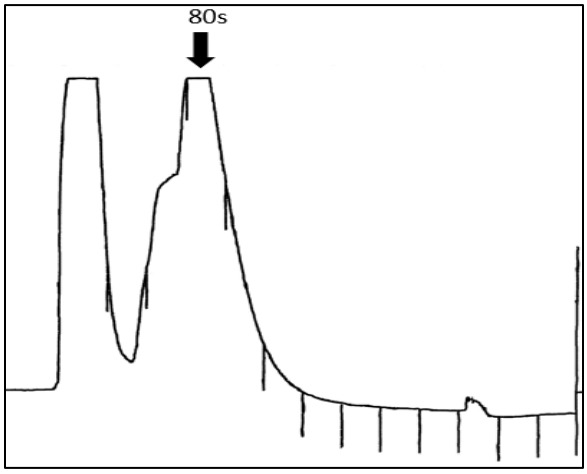
Figure 2.11: Translation rates and are not affected by loss of m6A. HEK-293T cells harvested after 72 hours transfection with METTL3/14 (M3/14) or negative control (Neg) siRNA and 24 hours of normoxic or hypoxic conditions. Polysome Profiling extracts separated by differential centrifugation followed by sucrose gradients. qRT-PCR analysis of fractions show percentage of individual mRNA. Error bars represent SEM of 3 experiments. Fraction containing 80S peak is marked.

re-oxygenation of HEK293T cells with and without METTL3/14 knockdown. Polysome profiles were obtained similarly to the previous experiments with the exception that cells were exposed to room level oxygen for 0.5, 1, or 4 hours prior to cyclohexamide treatment and fractionation of cellular extracts on sucrose gradients. Profiles (Figure 2.12) were again analyzed by qRT-PCR for our specific mRNA targets. After four hours of re-oxygenation, recovery of translational efficiency of Glut1, Jun, Dusp1, Hes1, and Myc was diminished by METTL3/14 knockdown compared to negative control siRNA transfected cells (Figure 2.13, Figure 2.14). These results suggest that m6A may be aiding the cellular response to recovery after stress.

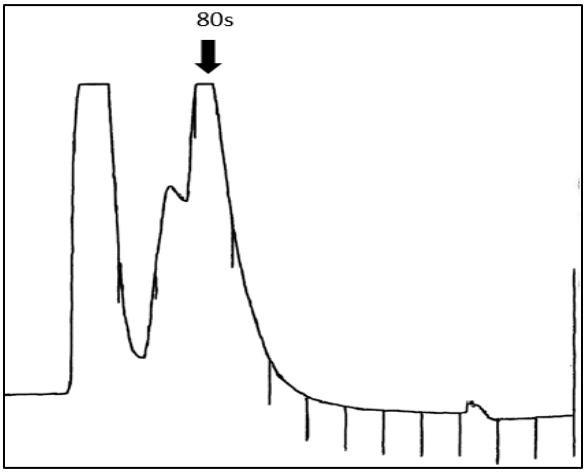
All of the mRNAs shift from heavier to lighter fractions in cells exposed to hypoxic conditions; however some mRNAs shifted farther into lighter fractions than others. For example, β2M and 18S shift completely out of polysomes into the sub-ribosomal fractions 2-3. However, Glut1 and Jun shifted only moderately from heavy fractions, with the majority of the mRNA being found in fractions 5 through 7. This initially suggested that Glut1 and Jun were still being moderately translated under hypoxia as has been previously reported [146]. However, it was also possible that mRNAs such as Glut1 and Jun had actually been released from the translating ribosomes, but were still in a large ribonucleoprotein complexes which might also increase their sedimentation in the gradient. To test whether the mRNAs were still bound by intact ribosomes and hence translating, extracts were treated with EDTA prior to sucrose sedimentation (Figure 2.10 E-F). EDTA chelates the magnesium necessary for large and small ribosomal subunit association, releasing all mRNAs to presumably sediment slower in their non-translating state. Interestingly, upon EDTA release, normoxic β2M shifted completely from heavy

Figure 2.12

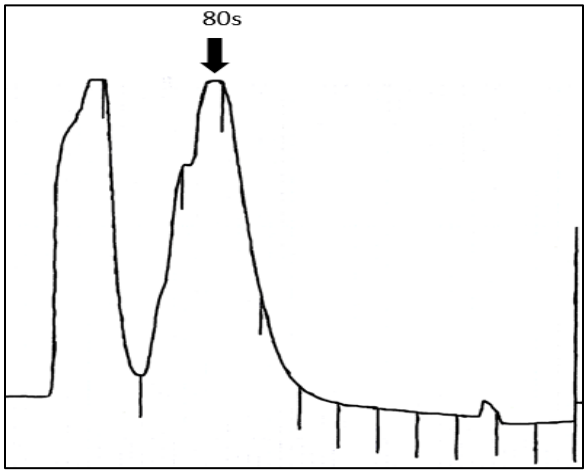
A.



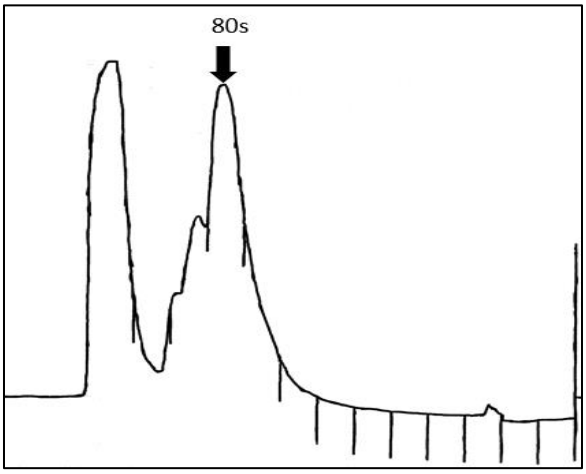
B.



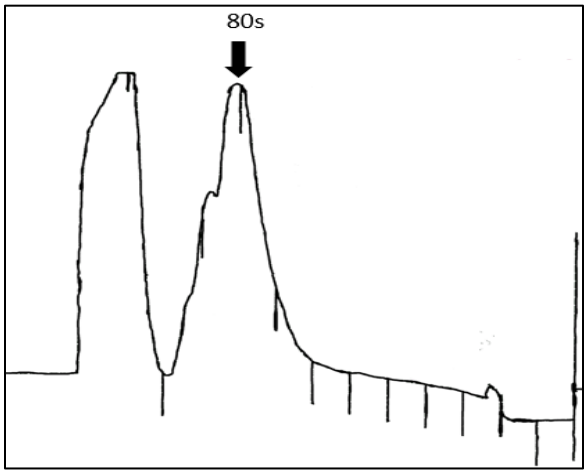
C.



D.



E.



F.

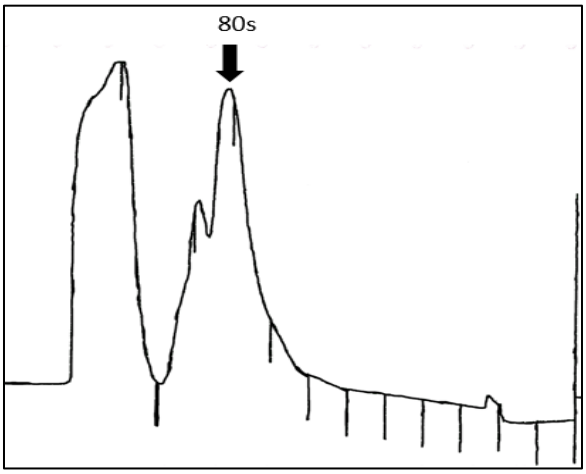


Figure 2.12: Reoxygenation Polysome Profile traces.

(A-F) Traces of Polysome Profiles from Figure 2.13. (A), 30min reoxygenation negative siRNA, (B), 30min reoxygenation m3/14 siRNA, (C), 1hr reoxygenation negative siRNA, (D), 1hr reoxygenation m3/14 siRNA, (E), 4hr reoxygenation negative siRNA, (F),4hr reoxygenation m3/14 siRNA. (Traces represent 1 of 3 experiments).

Figure 2.13

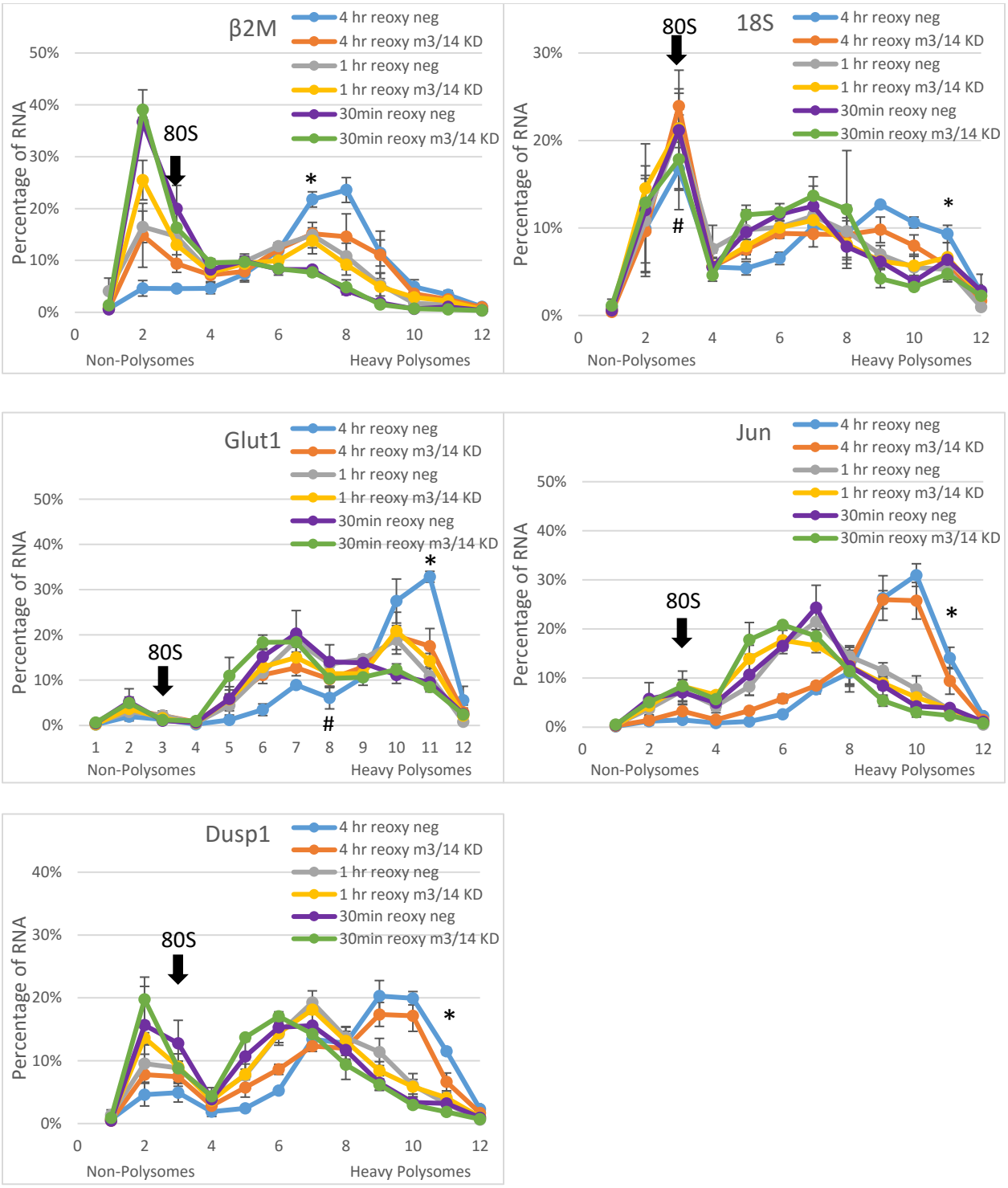

β2M
18S
Glut1
Jun
Dusp1
4 hr reoxy neg
4 hr reoxy m3/14 KD
1 hr reoxy neg
1 hr reoxy m3/14 KD
30min reoxy neg
30min reoxy m3/14 KD
Percentage of RNA
80S
Non-Polysomes
Heavy Polysomes
*
#

Figure 2.13: Mettl3/14 KD decreased the ability of messages to recover from hypoxia after 4 hours re-oxygenation. HEK-293T cells harvested after 72 hours transfection with Mettl3/14 (M3/14) or negative control (Neg) siRNA and 24 hours of hypoxia and either 0.5, 1 or 4 hours of room level re-oxygenation recovery. Polysome Profiling extracts separated by differential centrifugation followed by sucrose gradients. qRT-PCR analysis show percentage of individual mRNA in each fractions. Error bars represent SEM of 3 in the 1 and 4 hour time points and SEM of 2 in the 30 minute time point. Fraction containing 80S peak is marked.

Figure 2.14

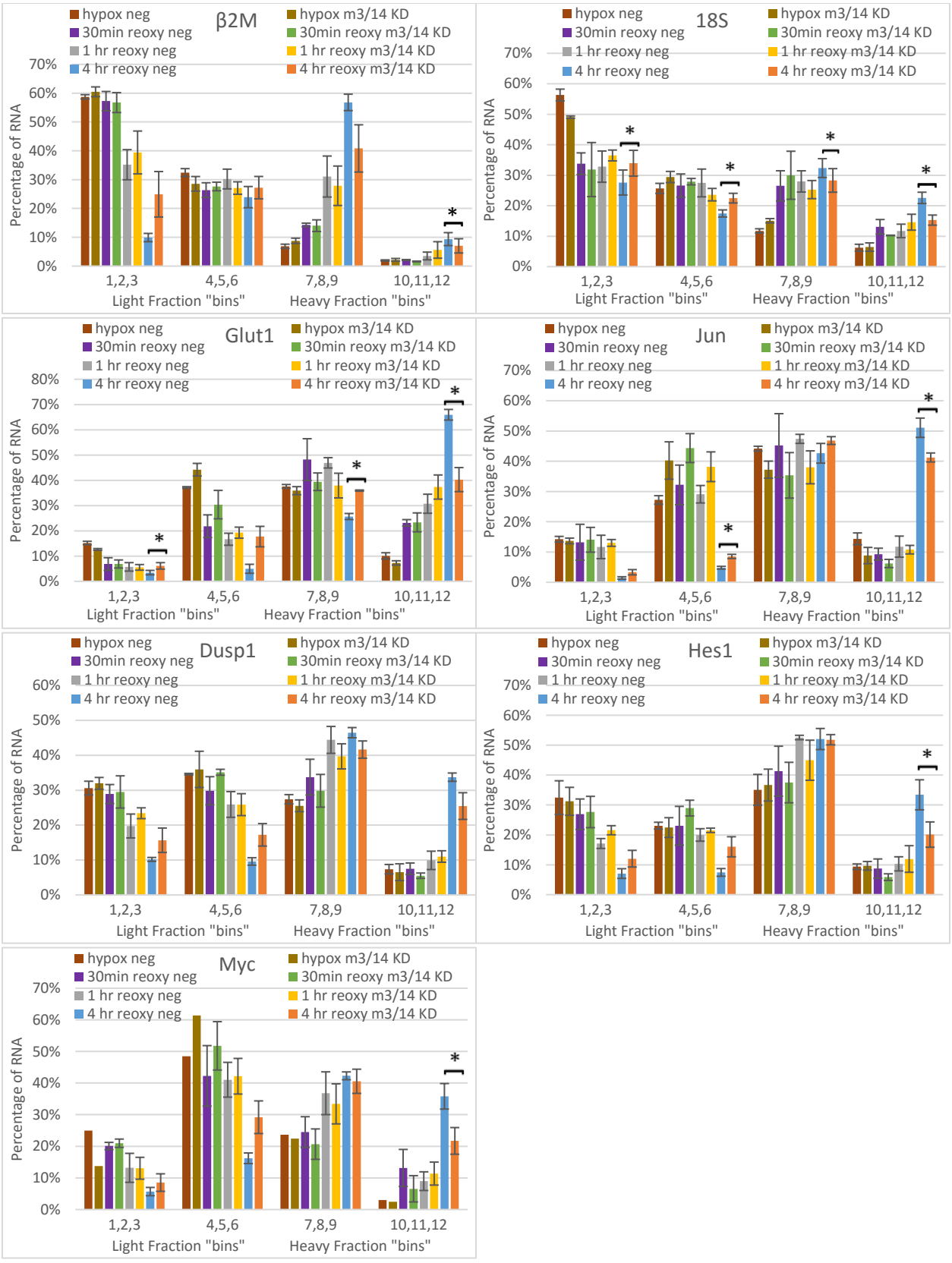

β2M
18S
Glut1
Jun
Dusp1
Hes1
Myc
hypox neg
30min reoxy neg
1 hr reoxy neg
4 hr reoxy neg
hypox m3/14 KD
30min reoxy m3/14 KD
1 hr reoxy m3/14 KD
4 hr reoxy m3/14 KD
Percentage of RNA
1,2,3
4,5,6
7,8,9
10,11,12
Light Fraction "bins"
Heavy Fraction "bins"

Figure 2.14. Mettl3/14 Knockdown decreased the ability of messages to recover from hypoxic stress after 4 hours re-oxygenation. HEK-293T cells harvested after 72 hours transfection with Mettl3/14 (M3/14 KD) or negative control (Neg) siRNA and 24 hours of hypoxic conditions and either 30 minutes, 1 hour or 4 hours of room level re-oxygenation recovery. Polysome Profiling of extracts separated by differential centrifugation followed by sucrose gradients. qRT-PCR analysis followed by binning into estimated non-polysome (1,2,3), light polysome (4,5,6), moderate polysome (7,8,9), and heavy polysome (10,11,12) bound fractions show percentage of individual mRNA in each bin. Error bars represent SEM of 3 experiments in the no re-oxygenation, 1 and 4 hour re-oxygenation experiments and SEM of 2 experiments in the 30 minute re-oxygenation experiment. N of 1 Myc hypoxia experiment is shown. *P ≤ 0.05 by Paired Student's t-test compared to binned negative control siRNA.

to light fractions just as observed with hypoxia with or without EDTA treatment (compare Figure 2.15 to Figure 2.11). However, Glut1 and Jun only shifted modestly from the heavier fractions to intermediate fractions, despite being released from ribosomes. The sedimentation of these mRNAs in intermediate fractions may indicate association with previously uncharacterized ribonucleoprotein complexes which are unaffected by EDTA metal chelation.

We attempted to correlate the mRNA translational efficiency to the steady-state level of their protein products. If m6A increases the stability of these mRNAs under hypoxic conditions, and knocking down m6A decreases their stability but maintains their translation, one might expect to see a decrease in their protein after m6A knockdown. However, western blotting for these proteins under normoxia and hypoxia after METTL3/14 knockdown showed no changes in protein levels of Glut1, Myc, and Dusp1 (Figure 2.8). There was also no observed changes in protein levels after re-oxygenation even though polysome analysis suggested that METTL3/14 knockdown decreased translational efficiency after 4 hours of recovery (Figure 2.16). This suggests that other factors likely are involved in determining the steady-state levels of proteins derived from these mRNAs. Interestingly, METTL3/14 knockdown also had no significant effect on HEK-293T cell proliferation under normoxic or hypoxic conditions (Figure 2.17).

Figure 2.15

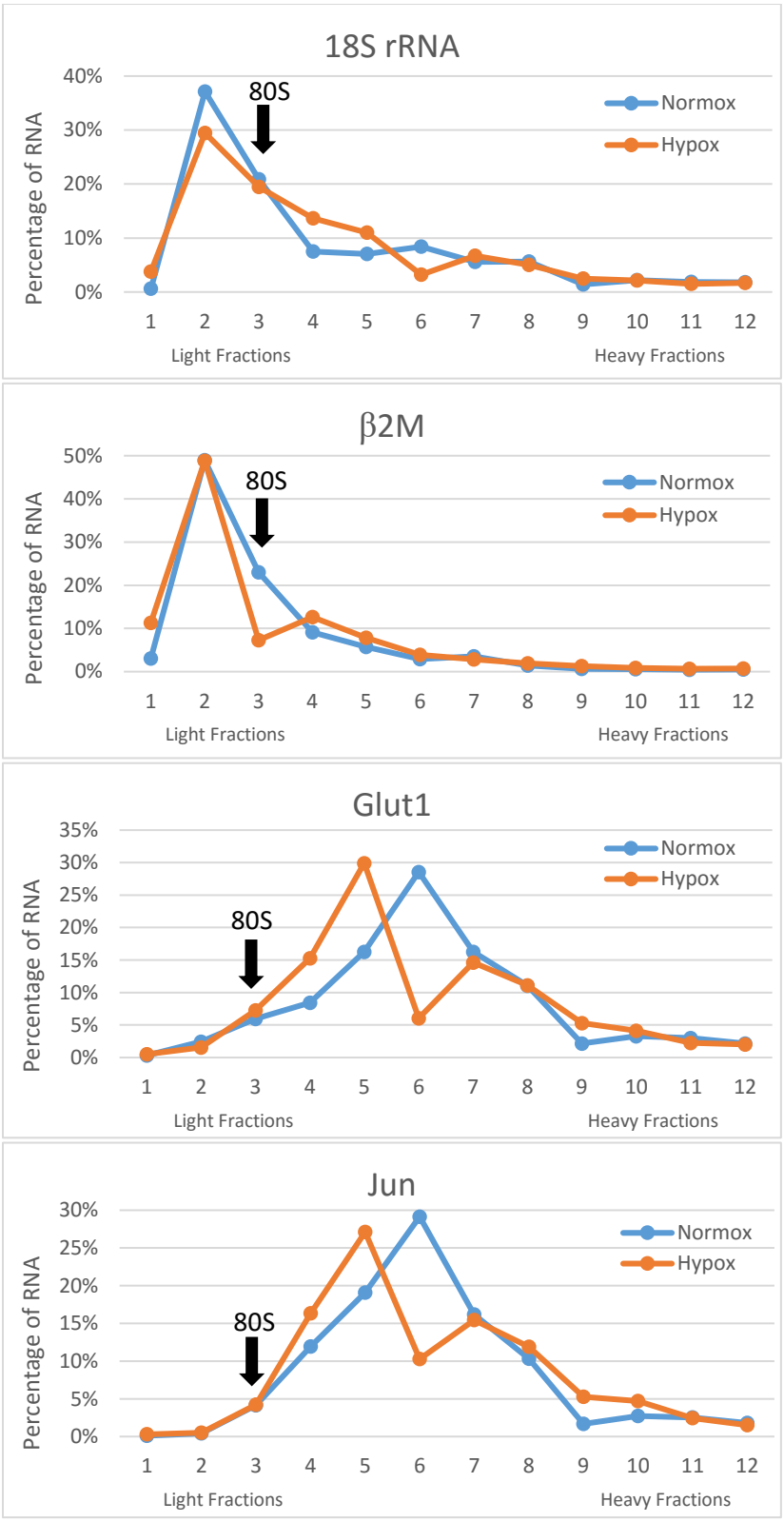

18S rRNA
Percentage of RNA
40%
30%
20%
10%
0%
80S
Normox
Hypox
1 2 3 4 5 6 7 8 9 10 11 12
Light Fractions
Heavy Fractions
β2M
Percentage of RNA
50%
40%
30%
20%
10%
0%
80S
Normox
Hypox
1 2 3 4 5 6 7 8 9 10 11 12
Light Fractions
Heavy Fractions
Glut1
Percentage of RNA
35%
30%
25%
20%
15%
10%
5%
0%
80S
Normox
Hypox
1 2 3 4 5 6 7 8 9 10 11 12
Light Fractions
Heavy Fractions
Jun
Percentage of RNA
30%
25%
20%
15%
10%
5%
0%
80S
Normox
Hypox
1 2 3 4 5 6 7 8 9 10 11 12
Light Fractions
Heavy Fractions

Figure 2.15: EDTA-resistant RNP particles are observed for several hypoxia-modified mRNAs.

Polysome Profiling following addition of 25mM EDTA added to the lysis buffer. qRT-PCR analysis of the fractions shows percentage of the specific mRNA in each fraction. Fraction containing 80S peak is marked. Compare to Figure 2.11.

Figure 2.16

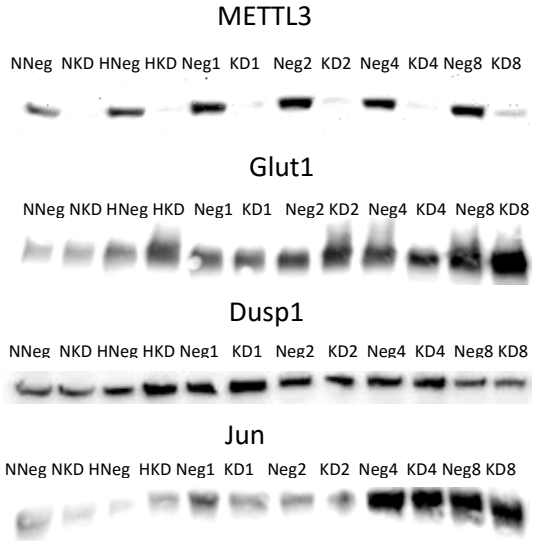

METTL3
NNeg NKD HNeg HKD Neg1 KD1 Neg2 KD2 Neg4 KD4 Neg8 KD8
Glut1
NNeg NKD HNeg HKD Neg1 KD1 Neg2 KD2 Neg4 KD4 Neg8 KD8
Dusp1
NNeg NKD HNeg HKD Neg1 KD1 Neg2 KD2 Neg4 KD4 Neg8 KD8
Jun
NNeg NKD HNeg HKD Neg1 KD1 Neg2 KD2 Neg4 KD4 Neg8 KD8

Figure 2.16: No observed changes in protein levels after re-oxygenation.

Western blots of 50 µg of protein lysates of normoxic negative control siRNA (NNeg), normoxic METTL3/14 Knockdown (NKD), hypoxic negative control (HNeg), hypoxic METTL3/14 Knockdown (HKD), and 1,2,4, or 8 hours re-oxygenation after hypoxia. (Representative of 3 experiments)

Figure 2.17

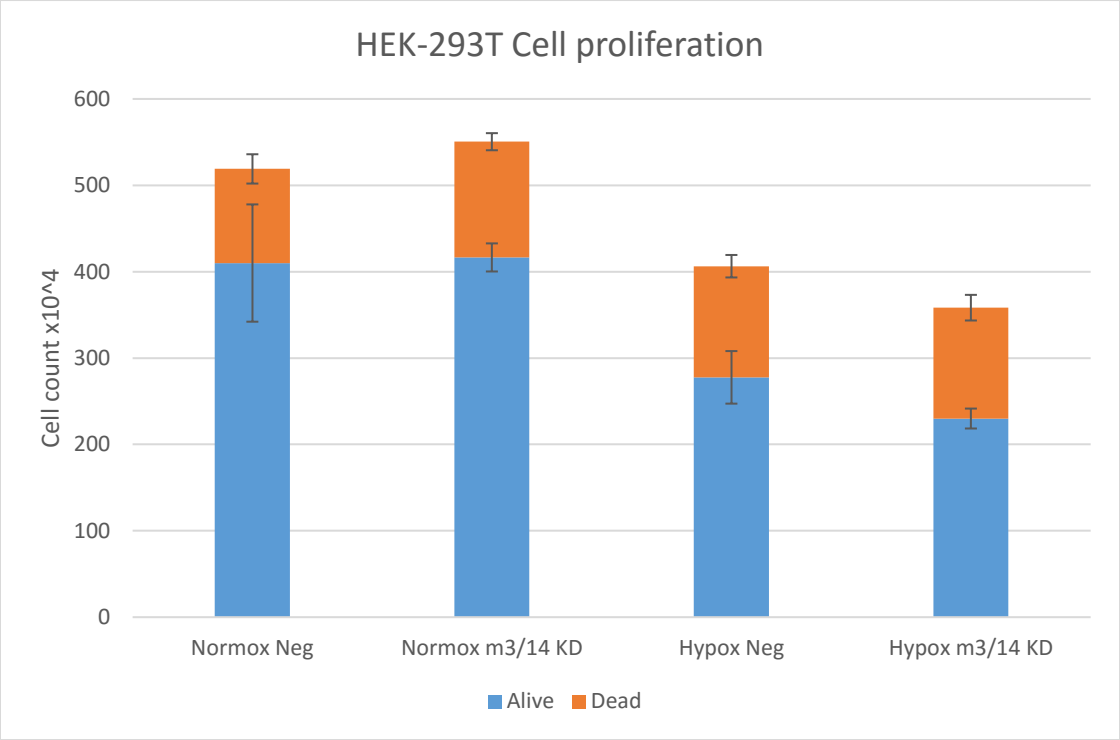

HEK-293T Cell proliferation
600
500
400
300
200
100
0
Cell count x10^4
Normox Neg
Normox m3/14 KD
Hypox Neg
Hypox m3/14 KD
Alive
Dead

Figure 2.17: HEK-293T cell proliferation after Mettl3/14 KD. Mettl3/14 Knockdown (m3/14 KD) had no significant effect on cell proliferation in HEK-293T cells grown in Normoxic (Normox) or 24 hour Hypoxic (Hypox) conditions when compared to negative control siRNA (Neg). (N of 3)

Summary

Our findings suggest that m6A mRNA methylation of certain mRNAs is induced by hypoxia. Furthermore, increased m6A prolongs the half-life of specific mRNA targets. Although, the increased stability of these messages did not correlate with translational efficiency or changes in protein levels during hypoxia, we found that loss of m6A through METTL3/14 knockdown decreased the cells ability to recover translational efficiency after re-oxygenation following hypoxic stress. We also observed that under hypoxic conditions some mRNAs may be associated with other ribonucleoprotein complexes instead of actively translating polysomes.

Discussion

m6A has received renewed attention over the past few years as a dynamic mRNA modification with many potential cellular functions. Although the factors involved in m6A mRNA methylation have been identified, the importance of mRNA methylation remains unclear. We now suggest a role for dynamic changes in mRNA m6A content in regulating mRNA stability in response to oxygen deprivation. In contrast to the total and rRNA, we saw a significant increase in the m6A content of mRNA levels in hypoxic cells, suggesting that the m6A modification may be important for regulating mRNAs in hypoxia. Our LC-MS/MS results also indicate the presence of other dynamic RNA modifications in PolyA$^+$, total and Ribosomal RNA after 24 hours of hypoxia, but we have yet to identify what the dynamic regulation of these modifications may signify.

Immunoprecipitation of m6A followed by qRT-PCR allowed us to determine the m6A methylation status of individual mRNAs. We observed an increase of m6A in specific mRNA targets after hypoxic exposure. However, this method cannot determine how hypoxia affects m6A methylation at specific sites. In follow up studies, we will seek to determine if hypoxia introduces m6A in new sites and if so, determine how specificity is regulated. It is possible that a hypoxic switch in methylation sites, for example switching methylation from the 5' end to the 3' end of the mRNA, could also alter the regulation of the mRNA without affecting the overall m6A level.

Others have previously reported that stability of individual mRNAs was increased by hypoxia [122, 124, 147-149]. It is also known that m6A can affect the stability of mRNAs [9, 13]. We now show that an increase in m6A methylation is correlated with increased stabilization of a number of mRNAs under hypoxic conditions. We confirmed the stabilization of Glut1 and Myc mRNA under hypoxia [124, 150] but also identified several novel targets including Dusp1, Hes1, and Jun. Interestingly, these findings contradict suggestions that increased methylation leads to increased degradation of mRNAs through YTHDF2 association [15]. The two data sets are not directly comparable however, as stability of those messages had not been reported under hypoxic conditions. There are a number of possible reasons for this discrepancy. Hypoxia may switch the location of the methylation allowing for different functions of the YTH family proteins, or equally possible, an entirely different RNA binding protein may be interacting with these hypoxically methylated mRNA. We will attempt to probe these ideas in future studies.

Even though m6A increased the stability of certain mRNAs under hypoxic conditions, there was no noticeable effect on translational efficiency or protein level. Polysome profiling detected no substantial changes in translation between samples containing or lacking METTL3/14. Remarkably, even though mRNA stability was increased, there was no detectable change in protein levels of Glut1, Myc, and Dusp1 as determined by western blotting. Interestingly, even though METTL3/14 knockdown did not affect translation, hypoxia itself caused a shift from heavier polysomes to a lighter complex. This shift to lighter fractions was more robust in some mRNAs than others. It was previously thought that this lack of a complete shift out of polysomes ultimately indicated the maintenance of translation. Indeed, it has previously been reported that numerous mRNAs including Hif-1α and Glut1 continue to associate with polysomes during hypoxic conditions [146, 151]. However, our data now suggest that these messages may be being maintained in mRNPs that do not contain 80S ribosomes, based on their resistance to EDTA-mediated disruption of ribosome association. None of the previous reports tested for this possibility.

Knockdown of METTL3/14 did decrease the ability of cells to recover translational efficiency after 4 hours of re-oxygenation following hypoxia. Detection of protein levels by western blotting after re-oxygenation again showed little difference with or without METTL3/14 KD, but polysome profiling showed decreases in translational efficiency after 4 hours of re-oxygenation in specific messages including Glut1, Hes1, Dusp1, and Myc suggesting that m6A's role in hypoxia may be related to recovery after the hypoxic stress rather than adaption to the stress. It is possible that the difference between these two data sets is due to differences in protein stability, or it is also

possible that we could not detect these subtle changes in protein levels accurately with western blotting. Utilizing a more physiologically relevant cell model or investigating this phenomenon in vivo might reveal situations in which m6A exerts a more dramatic effect in the adaptation to and recovery from hypoxic exposure.

Overall, this study demonstrates that hypoxic exposure can indeed induce changes in multiple RNA modifications. In particular, the m6A modification of mRNA is necessary for increased stability under hypoxic conditions. Future studies will explore these m6A changes with base-specific precision, as well as the RNA binding proteins that interact with the m6A modification under hypoxia. It is our goal to gain a better understanding of mRNA dynamics in response to hypoxia with the hope of developing new therapeutics targeting cardiovascular disease, cancer and other diseases that involve periods of reduced oxygen.

Materials and Methods

Cell Lines

HEK293T (HEK293T/17; CRL-11268) cells were obtained directly from ATCC (Manassas, VA) and maintained in high glucose (4g/L) DMEM (Corning/Mediatech, Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 2mM Glutamine (Corning/Mediatech), and 1X Pen/Strep (Corning/Mediatech) and passaged when approximately 85-90% confluent. Cells were tested for mycoplasma upon receipt. For experiments, cells were plated on 10cm dishes (CytoOne, USA Scientific, Orlando, FL) in high glucose (4.0 g/L) media and allowed to attach/recover for

18-24 hours. The next day, the media was removed and replaced with media containing 1 g/L glucose. Hypoxic treatments were carried out in a Ruskin In Vivo 400 Hypoxia Hood (The Baker Company, Sanford, ME) maintained at 37°C, 5% CO_2, 70% humidity and 1% oxygen. All other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

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). For RNA extraction from ribonucleoprotein immunoprecipitations (RNP-IP) and sucrose gradients, GlycoBlue (Life Technologies) was added as a carrier during the precipitation step. RNA quality and quantity was determined via NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA).

PolyA$^+$ RNA purification

PolyA$^+$ 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.

LC-MS/MS of PolyA$^+$ RNA for m6A modification analysis

PolyA$^+$ RNA was hydrolyzed enzymatically by first denaturing the RNA at 95ºC followed by immediate placement on ice. PolyA$^+$ RNA was incubated with S1 nuclease buffer and 1 unit of S1 nuclease (Life Technologies) per 300 ng of RNA for 30 minutes at 37ºC. Alkaline phosphatase buffer, 1 unit of Alkaline Phosphatase per 300 ng of RNA (Life Technologies), and .00025 units of venom phosphodiesterase I (Sigma-Aldrich) was added to incubate for 30 minutes at 37ºC. Fragmented RNA was purified through two round of chloroform extraction. LC-MS/MS quantification of m6A and adenine was performed by Craft Technologies (Wilson, NC). Separations and identification by LC-MS/MS were performed using a Thermo Finnigan Linear Ion Trap Quadrapole (LTQ) mass spectrometer utilizing an electrospray ionization interface in selected reaction monitoring mode connected to Agilent 1100 autosampler and Agilent 1100 HPLC pump system (Agilent). Detection was performed using an electrospray ionization source operated in positive ion mode.

LC-MS/MS of PolyA$^+$, Total, and Ribosomal RNA for nucleoside modification analysis

Purified RNA was digested to individual nucleosides and modified nucleosides were quantified as previously described [36]. Briefly, digestion was performed with nuclease P1 (Sigma, 2U) in buffer containing 25 mM NaCl and 2.5 mM ZnCl2 for 2h at 37ºC, followed by incubation with Antarctic Phosphatase (NEB, 5U) for an additional 2h at 37ºC. Nucleosides were then separated and quantified at the Duke Molecular Physiology Institute using UPLC-MS/MS as previously described [152], except acetic acid replaced formic acid in the mobile phase.

Ribosomal Subunit Separation

Cells grown in normoxic or hypoxic conditions were harvested in “Buffer A” (35mM Tris pH 7.5, 70mM KCL, 9mM MgCl, 0.1 mM EDTA, 250mM sucrose, 0.5% Sodium Deoxycholate, 1% Triton X 100, 1x Protease Inhibitors, 1mM DTT, and RNase out). Cell lysate was centrifuged for 15 minutes at 15,000 x g in a Beckman TLA 100 rotor. The supernatant was removed to a new tube and centrifuged in the same rotor at 150,000 x g for 90 minutes. Ribosome pellets were resuspended in “Buffer B” (10mM Tris pH 7.5, 500mM KCL, 10mM $MgCl_2$ 1x Protease Inhibitors 1mM DTT and RNase out) and layered on a 12mL 15-30% sucrose gradient in buffer B. The gradient was centrifuged at 86,000 x g for 14 hours in a Beckman SW-41 Ti swinging bucket rotor. 1 mL fractions were collected from the top of the gradient and the positions of the 40S and 60S ribosomal subunits were found by measuring each fraction at an absorbance at 254 nm. RNA was isolated from each sample via Trizol.

m6A mRNA Immunoprecipitation (MeRIP)

m6A Ribonucleoprotein Immunoprecipitation reactions were performed by first isolating $PolyA^+$ RNA from normoxic and hypoxic cells. Protein G Dynabeads (Thermo Fisher Scientific, Baltics UAB) were washed 3X in 1 mL of IPP buffer (10mM Tris-HCL pH7.4, 150mM NaCl, 0.1% NP-40). 25 µl of beads required per IP. Anti-N6-methyladenosine mouse monoclonal antibody (EMD Millipore, Temecula, CA, MABE1006) was added to the beads (5 µg/IP) and brought up to 1mL with IPP buffer. As a negative control, beads without antibody were used as well. Bead mixture was tumbled for 16 hours at 4ºC.

Beads were washed 5X with IPP buffer and 100ng of PolyA$^+$ RNA was added to the beads along with 1mM DTT and RNase out. The mixture was brought up to 500 µl with IPP buffer. Bead mixture was tumbled at 4ºC for 4 hours. Beads were washed 2X in IPP buffer, placed into a fresh tube, and washed 3X more in IPP buffer. m6A RNA was eluted off the beads by tumbling 2X with 125 µl of 25mg/mL N^6-Methyladenosine-5'-monophosphate sodium salt (CHEM-IMPEX INT'L INC., Wood Dale, IL). Supernatant was added to Trizol-LS followed by RNA isolation as per manufacture's protocol. Final RNA sample was brought up in 10 µl of water.

PCR for MeRIP

Reverse transcription was performed on 10 µl m6A PolyA$^+$ RNA from the MeRIP with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). After diluting cDNA two-fold, quantitative real-time PCR was performed using a Roche Lightcycler 96 with Fast Start Essential DNA Green (Roche Diagnostics Corporation, Indianapolis, IN) and primers from Integrated DNA Technologies, Inc. (Coralville, Iowa). Primer efficiency was verified to be over 95% for all primer sets used. Quantification of mRNA from the MeRIP was carried out via ΔΔCT analysis against non-immunoprecipitated input RNA and RNA pulled down from non-antibody bound beads. All real-time PCR primer sets were designed so the products would span at least one intron (>1kb when possible), and amplification of a single product was confirmed by agarose gel visualization and/or melting curve analysis.

siRNA Transfections

Either a negative siRNA (Silencer; Life Technologies, Carlsbad, CA) or METTL3 and METTL14 siRNAs (Qiagen, Germantown, MD) transfected together using Lipofectamine RNAi Max 54 µl/plate as per manufacturer's protocol (Life Technologies) using 180 pM siRNA/10 cm dish. Cells were incubated for 48-72 hours post-transfection with the last 24 hours in either normoxic or hypoxic conditions.

4SU

mRNA half-life determinations using 4SU were performed as per established protocol [153]. Cells were treated with 200 µM 4SU (Sigma-Aldrich) for 1 hour. RNA isolated via Trizol was biotinylated by labeling 50 µg RNA in a reaction mixture with 50 µl 10x Tris/EDTA buffer (TE), 100 µl 1mg/ml Biotin-HPDP (EZ-Link Biotin HPDP, Thermo Scientific, Waltham, MA) in Dimethylformamide (DMF), and RNase free H_2O brought to 400 µl. Mixture was incubated in the dark with rotation for 1.5 hours. Biotinylated RNA was extracted using an equal volume of Chloroform/Isoamyl alcohol (24:1) 2x in phase lock gel heavy tubes (5 Prime, Gaithersburg, MD) followed by RNA precipitation with isopropanol. RNA was heated to 65ºC for ten minutes and placed immediately on ice. RNA was added to Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific) that had been thoroughly washed and resuspended in 2X streptavidin binding buffer (2X TE, 2M NaCl). The RNA bead mixture was incubated with rotation for 30 minutes. Beads were washed 5X with 65ºC Wash Buffer (1XTE, 1M NaCl, 0.1% Tween20) and the supernatant was kept containing the non-labeled RNA. Three rounds of 100 mM Dithiothreitol (DTT) elution followed by 1 round of TE eluted labeled RNA from the beads. RNA was isolated via Isopropanol and resuspended in 40 µl of water.

mRNA half-life

mRNA levels were determined by real-time quantitative PCR using 1 µl of RNA from both labeled and unlabeled 4SU samples. Decay rates were calculated by the natural log of 1 minus the RNA input normalized ratio of labeled over unlabeled RNA. All half-lives were normalized to GAPDH half-life which was defined as 8 hours based on previous publications and our unpublished data [154].

Polysome Profiling

Cells were treated with 200 µM cyclohexamide for 15 minutes prior to harvest. Cells were harvested in PLB buffer (100mM KCl, 5mM $MgCl_2$, 10mM Hepes, 0.5% NP40, 200 µM cyclohexamide, 1mM DTT, 1x Protease Inhibitors, RNase out) and incubated on ice for 30 minutes. Lysates were pre-cleared by centrifugation for 8 minutes at 5000 x g. Supernatant was layered on a 10-45% sucrose gradient in Polysome Profile Buffer (300 mM KCl, 50mM Hepes, 10mM $MgCl_2$, 200 µM cyclohexamide). The gradient was centrifuged at 38,000 x g for 1 hour 45 minutes in a Beckman SW-41 Ti swinging bucket rotor. Twelve 1 mL fractions were collected from the top of the gradient and the polysomes were measured from lightest to heaviest at an absorbance at 254 nm. RNA was isolated from each sample via Trizol.

Profiling PCR

Reverse transcription was performed on 1 µl of RNA from each fraction in a 20 µl reaction. After diluting cDNA five-fold, quantitative real-time PCR was performed.

Percentages of mRNA per fraction was carried out via ΔCT analysis using a baseline Cq value representing 0% mRNA. Percentage of ribosomal RNA was calculated in the same manner.

Western Blots

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 (30-50 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). Transfer was verified via Ponceau S staining then 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 HRP-linked secondary antibody (GE Healthcare), washed again with TBST, developed using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA), and exposed to film for detection.

Statistical Analysis

All experiments were performed on at least three separate occasions to generate biological replicates unless otherwise indicated. qPCR was performed at least twice on each cDNA for technical verification of data. Half-lives were calculated for each biological replicate and then averaged together to determine final value and standard

error of the mean. Statistical significance was calculated by a two-tailed, paired Student's t-test comparing experimental to control conditions. A P-value below 0.05 was defined as statistically significant.

Primer List

Gene	Forward (5'-3')	Reverse (5'-3')
18S rRNA	CTGAGAAACGGCTACCACATC	GCCTCGAAAGAGTCCTGTATTG
28S rRNA	GGGTGGTAAACTCCATCTAAGG	GCCCTCTTGAACTCTCTCTTC
5.8S rRNA	CTCGTGCGTCGATGAAGAA	TCGAAGTGTCGATGATCAATGT
5S rRNA	CGTCTGATCTCGGAAGCTAAG	CCTACAGCACCCGGTATTC
β2M	AGATGTCTCGCTCCGTGGCCTTA	TGTCGGATGGATGAAACCCAGACA
Dusp1	CAACCACAAGGCAGACATCA	CAGTGGACAAACACCCTTCC
eEF1A1	CGGTCTCAGAACTGTTTGTTTC	AAACCAAAGTGGTCCACAAA
GapDH	AAGGTCGGAGTCAACGGATTTGGT	AGCCTTGACGGTGCCATGGAATTT
Glut1	TATCGTCAACACGGCCTTCACTGT	CACAAAGCCAAAGATGGCCACGAT
Hes1	GAAGGCGGACATTCTGGAAAT	GTCACCTCGTTCATGCACTC
HuR	CCTGTTCAGCAGCATTGGTGAAGT	TTCAGCGTGTTGATCGCTCTCTCT
Jun	TTCTATGACGATGCCCTCAAC	TCAGGGTCATGCTCTGTTTC
METTL3	AGCCTTCTGAACCAACAGTCC	CCGACCTCGAGAGCGAAAT
Myc	TCCTCGGATTCTCTGCTCTCCT	AGAAGGTGATCCAGACTCTGACCT
VHL	TCTCTCAATGTTGACGGACAGCCT	GGTCTTTCTGCACATTTGGGTGGT

siRNA's

siRNA	Target Sequence
siMETTL3	CTGCAAGTATGTTCACTATGA
siMETTL14	AAGGATGAGTTAATAGCTAAA
Negative Control #1 siRNA	Ambion cat. # AM4635

Antibodies

Antibody	Catalogue #	Vendor	WB Dilution	IP Amount
c-Jun	MA5-15119	Thermo Fisher	1/100	
c-Myc	NBP1-19671	Novus Biologicals	1/1000	
GAPDH (G-9)	sc-365062	Santa Cruz Biotechnology	1/100	
Glut1	PA5-16793	Thermo Fisher	1/250	
METTL14	HPA038002	Sigma Life Science	1/1000	
METTL3/MT-A70	A301-567A	Bethyl Laboratories	1/5000	
MKP-1 (c-19) (DUSP1)	sc-370	Santa Cruz Biotechnology	1/100	
N6-methyladenosine (m6a)	MABE1006	EMD Millipore		5 µg

Chapter 3

N^6-methyladenosine in breast cancer progression

ABSTRACT

The mRNA modification, N^6-methyladenosine (m6A), has recently been shown to be involved in many post-transcriptional regulation processes including mRNA stability and translational efficiency. While understanding these mechanisms are useful, it is also imperative to correlate these processes with phenotypic outputs. Here we report that m6A levels are decreased in genetically defined immortalized and oncogenically transformed human mammary epithelial cells (HMECs) as compared with their primary cell predecessor. Interestingly, after 24 hours of hypoxic exposure, m6A levels in the immortal and transformed cells were increased back to primary cell levels. It was also found that the m6A methyltransferase, Mettl3, is decreased and the demethylase, Alkbh5, is increased in the immortalized and transformed cell lines, possibly explaining the decrease in m6A in those cells. However, changes in Mettl3 and Alkbh5 do not explain the increase in m6A in hypoxia. m6A levels appear increased in hypoxic conditions not because of changes in methyltransferase or demethylase levels, but rather because of a build-up of stabilized m6A mRNA. At first glance, it would appear that transformation of the HMECs reduces m6A levels possibly to obtain a more progressive phenotype. In actuality, increasing m6A levels through overexpression of Mettl3 and Mettl14 increased proliferation, migration, and invasion of the transformed cells. Remarkably, overexpression of Mettl3 and Mettl14 had little effect on the immortalized cells, suggesting that the m6A modification may regulate migration and invasion differently in cells depending on their oncogenic potential.

Introduction

It is estimated that there will be over 250,000 new cases of breast cancer in the United States in 2017, and over 40,000 Americans are expected to lose their lives to breast cancer in 2017 [155]. The 5 year survival rate for patients diagnosed with localized breast cancer is nearly 99%. Unfortunately, patients with stage IV metastatic breast cancer have less than a 30% survivability rate [156, 157]. Gene expression profiles of breast cancer have been extensively studied, but more progress needs to be made for early detection and proper personalized treatment for the disease. Because of this, it is important to study post-transcriptional and translational pathways that regulate *de novo* gene expression in breast cancer cells with the eventual goal of identifying better therapeutic targets for suppressing growth and metastasis of tumor cells.

Recently, the mRNA modification, m6A has been shown to be involved in many post-transcriptional regulation processes. It has been discovered that m6A levels are directly correlated to metastasis and poor patient prognosis in hepatocellular carcinoma (HCC). In fact, a decrease in m6A content led to increased metastasis of HCCs [91]. Interestingly, loss of m6A through an increase in ALKBH5, an m6A demethylase, also led to enhanced breast and glioblastoma cancer stem cell self-renewal and growth [15, 86, 87]. Inversely, an increase in the m6A methyltransferase, Mettl3, led to increased invasion of lung adenocarcinoma cells [38]. While it is clear that the function of m6A is diverse throughout many cell types, it is also important to understand the role that the m6A modification plays in all tumors.

Because tumors often quickly outgrow their blood supply, they have to adapt to the hypoxic conditions. Hypoxic breast cancer cells adapt to these conditions through

HIF mediated angiogenesis [158]. Not only does HIF increase vascularization of the tumor to increase blood and oxygen supply, but it is also known to promote metastasis of the cells [159-161]. Interestingly, ALKBH5, an m6A demethylase, is also regulated by HIF [162]. Recently, it was reported that a HIF-regulated decrease in m6A through an increase in ALKBH5 and or ZNF217 maintains pluripotency of breast cancer stem cells in a number of established breast cancer cell lines [15, 88]. Interestingly, we recently reported that hypoxia led to an increase in m6A mRNA levels in HEK-293T cells ultimately leading to increased stability and recovery of translational efficiency after reoxygenation [11]. Because hypoxia both regulates m6A levels and promotes metastasis in breast cancer cells, it is important to understand if m6A might have a role in hypoxia mediated breast cancer metastasis.

The m6A modification is the most abundant modification in mRNA [4]. This modification has been shown to be important for the stability and translational efficiency of mRNA [6-11], and is involved in the pluripotency of stem cells in embryonic development [12-14]. Methyltransferase like -3 and -14, as well as Wilms' tumor associating protein (WTAP) form the m6A methyltransferase complex which methylates nascent pre-mRNA within the nucleus [17-21]. The enzymatically active component of the methyltransferase, Mettl3, contains an S-adenosyl methionine (SAM) binding domain, and utilizes SAM as a substrate to methylate target mRNAs that contain a DRACH m6A consensus sequence, often found in 3` UTR's and around both start and stop codons [17, 24-27]. METTL14 lacks catalytic activity but participates in mRNA binding/targeting [28-30]. WTAP is responsible for the localization of the Mettl3/14 complex to the nuclear speckle, and greatly enhances methyltransferase activity by

bringing the methyltransferase to the pre-mRNA. m6A methylation of mRNA is reversible and can be reportedly removed by alkylation repair homolog 5 (ALKBH5) and fat mass and obesity related protein (FTO) [39-48]. However, recent data suggests that FTO prefers the modification N^6,2′-*O*-dimethyladenosine (m6Am) as a substrate rather than m6A [53].

Methylated mRNA is transported out of the nucleus and bound by the YTH family RNA binding proteins, including YTHDF1, YTHDF2, and YTHDC1 [9, 56, 57]. The broader consequences of RNA methylation through the actions of these and other RNA binding proteins are still being investigated. However, YTHDF2 has been shown facilitate degradation of methylated mRNAs by transporting them to P bodies [9, 23, 40, 61]. Alternatively, binding of YTHDF1 increases translational efficiency of m6A methylated mRNA [10]. Lastly, YTHDC1 recruits splicing factors to regulate splicing of m6A methylated mRNA [64]. The interactions between these RNA binding proteins is not fully understood, and competition between them may yield different results for the mRNA and ultimately for the protein output.

m6A methylation has recently been correlated with a number of phenotypic changes in cancers including in breast cancer stem cells. Many of these phenotypic changes are the result of changing protein expression of either the m6A methyltransferases, demethylases or RNA binding proteins. These studies show that m6A has a functional significance in cancer, but further investigation is required in order to understand the intricacies of the m6A mechanisms when compared with non-cancerous cells. In this study, we hypothesize that m6A methylation plays a role in the progression of normal to cancer states.

Results

Because diagnosing breast cancer early while it is still localized provides a much better outcome and survival rate, it is important to fully understand breast cancer progression. However, because cancers have many diverse mutations and alterations to gene regulation, it is difficult to pinpoint exactly which changes introduce aggressive phenotypic behavior. For this reason, we chose to use a genetically defined breast cancer progression model for these studies. In this model, three cell types are utilized, primary Human Mammary Epithelial cells (HMECs), HMECs immortalized through the stable expression of hTERT, $p53^{DD}$, cyclin D1, $CDK4^{R24C}$, and $C\text{-}MYC^{T58A}$, and a further oncogenically transformed line expressing with $H\text{-}RAS^{G12V}$ in addition to the above alterations (Figure 3.1) [163]. hTERT is the catalytic subunit of telomerase which helps the cells avoid senescence, resulting in a potentially unlimited lifespan. The $p53^{DD}$ construct expresses a dominant negative form of the tumor suppressor p53 lacking DNA binding and transactivation domain that inhibits tetramerization and prevents it from regulating the cell cycle, apoptosis, and differentiation. This effectively nullifies cellular checkpoints in response to DNA damage, etc. The $CDK4^{R24C}$ mutant is resistant to inhibition and along with CyclinD1 overexpression leads to hyperphosphorylation of Rb further disrupting cell cycle checkpoint function. The T58A mutation of the proto-oncogene CMYC confers resistance to degradation by preventing threonine phosphorylation. This promotes cellular proliferation as well as altered metabolism. Finally, the $H\text{-}RAS^{G12V}$ oncogene supports growth factor-independent cell proliferation and promotes survival, invasion, angiogenesis, and metastasis.

We first investigated whether m6A levels were altered in our breast cancer progression model, and what effect hypoxia had on m6A in the HMEC cell lines. HMEC primary, immortalized, and oncogenically transformed cells were incubated for 24 hours under normoxic or hypoxic (1% O_2) conditions. PolyA$^+$ mRNA was isolated by oligo-dT selection followed by ribosomal RNA (rRNA) depletion, and after fragmentation, liquid chromatography and tandem mass spectrometry (LC-MS/MS) were used to quantify various RNA modifications in the mRNA enriched samples. Interestingly, when compared to the primary HMECs, m6A levels were decreased in the immortalized and transformed cell lines under normal growing conditions. However, in hypoxic conditions the m6A levels in the immortal and transformed lines were increased to levels comparable to the primary cells, which surprisingly showed no change (Figure 3.2). Many other mRNA modifications, including 5-methylcytidine and 2-O-methyladenosine were unchanged in the immortalized and transformed cells, further highlighting the need to understand why m6A levels are specifically changed (Figure 3.3).

Protein Levels of m6A methyltransferases and demethylases

In order to investigate the dynamic response of m6A to cellular transformation as well as hypoxia, RNA and protein levels of the m6A-associated enzymes and effectors were measured through qRT-PCR and Western Blotting following SDS-PAGE. . Interestingly, many of the proteins involved with m6A, including methyltransferases, demethylases, and RNA binding proteins exhibited notable changes upon immortalization and oncogenic transformation of the HMEC cells. Hypoxia, however, had very little effect on protein levels. Fascinatingly, the mRNA levels did not correspond with the protein levels of many of these targets (Figure 3.4).

Figure 3.1

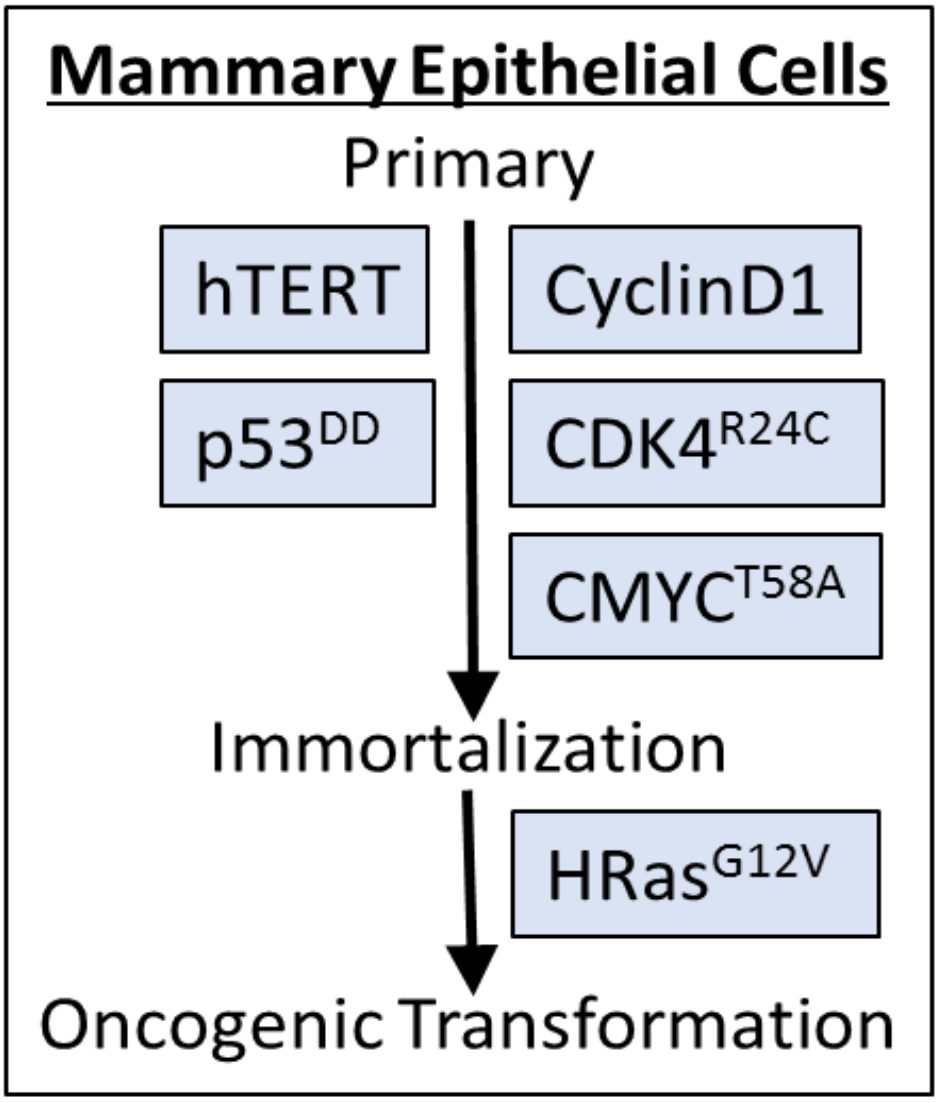

Mammary Epithelial Cells
Primary
hTERT
CyclinD1
p53DD
CDK4R24C
CMYCT58A
Immortalization
HRasG12V
Oncogenic Transformation

Figure 3.1: HMEC cancer progression cell lines. Outline of the cellular transformation in the genetically defined human mammary epithelial cancer cells. In this model, three cell types are utilized, primary Human Mammary Epithelial cells (HMECs), HMECs immortalized through the stable expression of hTERT, $p53^{DD}$, cyclin D1, $CDK4^{R24C}$, and $C\text{-}MYC^{T58A}$, and a further oncogenically transformed line expressing with $H\text{-}RAS^{G12V}$ in addition to the above alterations

Figure 3.2

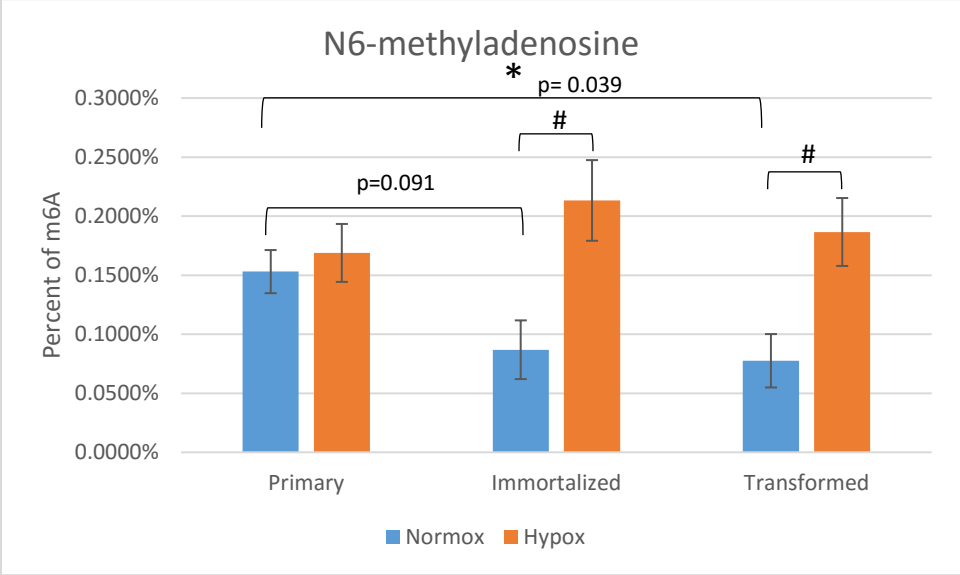

N6-methyladenosine
*
p= 0.039
#
#
p=0.091
0.3000%
0.2500%
0.2000%
0.1500%
0.1000%
0.0500%
0.0000%
Percent of m6A
Primary
Immortalized
Transformed
Normox
Hypox

Figure 3.2: m6A decreases in immortalized and oncogenically transformed cells in normoxic but not hypoxic conditions.

LC-MS/MS determination of m6A levels in mRNA isolated from primary, immortalized, and oncogenically transformed (transformed) HMEC cells grown in Normoxic (Normox) or Hypoxic (Hypox) conditions for 24 hours. Values represent the amount of m6A divided by total Adenosine (N of 3). *P ≤ 0.05 by Paired Student's t-test. Error bars represent standard error of the mean (SEM).

Figure 3.3

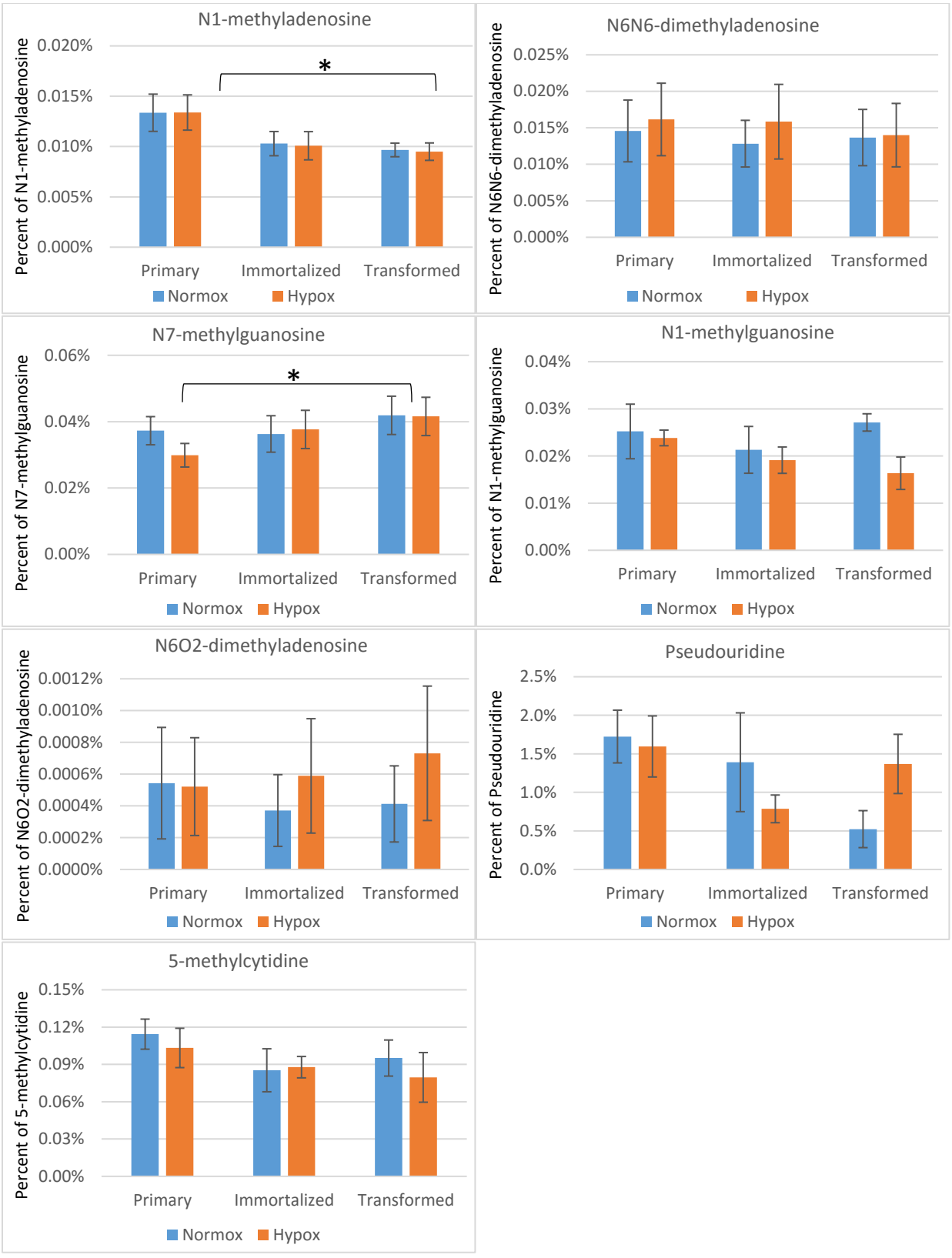

N1-methyladenosine
Percent of N1-methyladenosine
0.020%
0.015%
0.010%
0.005%
0.000%
*
Primary
Immortalized
Transformed
Normox
Hypox
N6N6-dimethyladenosine
Percent of N6N6-dimethyladenosine
0.025%
0.020%
0.015%
0.010%
0.005%
0.000%
Primary
Immortalized
Transformed
Normox
Hypox
N7-methylguanosine
Percent of N7-methylguanosine
0.06%
0.04%
0.02%
0.00%
*
Primary
Immortalized
Transformed
Normox
Hypox
N1-methylguanosine
Percent of N1-methylguanosine
0.04%
0.03%
0.02%
0.01%
0.00%
Primary
Immortalized
Transformed
Normox
Hypox
N6O2-dimethyladenosine
Percent of N6O2-dimethyladenosine
0.0012%
0.0010%
0.0008%
0.0006%
0.0004%
0.0002%
0.0000%
Primary
Immortalized
Transformed
Normox
Hypox
Pseudouridine
Percent of Pseudouridine
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Primary
Immortalized
Transformed
Normox
Hypox
5-methylcytidine
Percent of 5-methylcytidine
0.15%
0.12%
0.09%
0.06%
0.03%
0.00%
Primary
Immortalized
Transformed
Normox
Hypox

Figure 3.3: UPLC-MS/MS of Poly A$^+$ RNA from HMEC cells cultured in Normoxic or Hypoxic conditions for 24 hours. Values represent the amount of the modification divided by total parent levels (N of 3). *P ≤ 0.05 by Paired Student's t-test. Error bars represent Standard error of the mean (SEM).

Figure 3.4

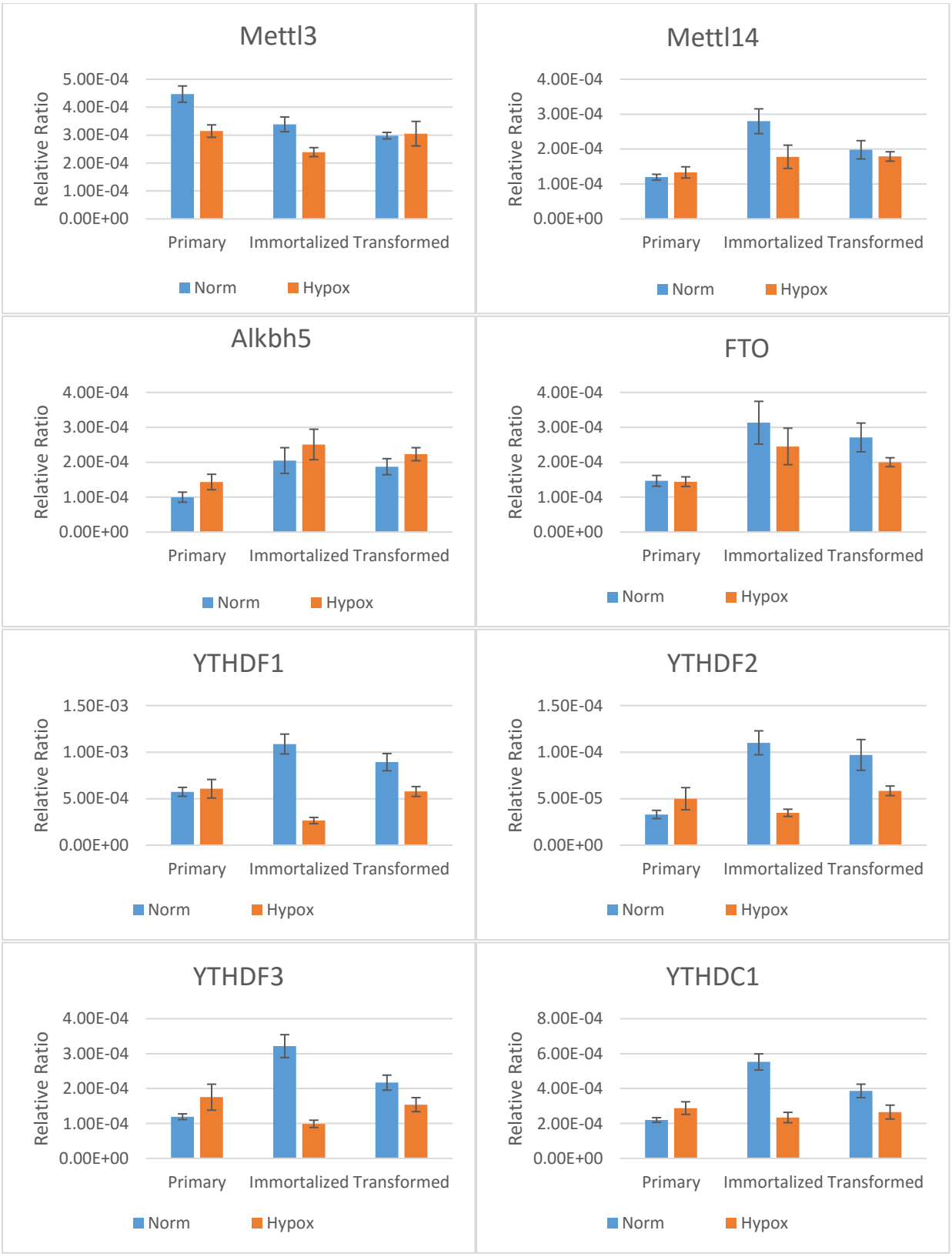

Mettl3
Relative Ratio
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox
Mettl14
Relative Ratio
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox
Alkbh5
Relative Ratio
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox
FTO
Relative Ratio
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox
YTHDF1
Relative Ratio
1.50E-03
1.00E-03
5.00E-04
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox
YTHDF2
Relative Ratio
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox
YTHDF3
Relative Ratio
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox
YTHDC1
Relative Ratio
8.00E-04
6.00E-04
4.00E-04
2.00E-04
0.00E+00
Primary
Immortalized
Transformed
Norm
Hypox

Figure 3.4: qRT-PCR of m6A players qRT-PCR of m6A methyltransferases, demethylases, and m6A RNA binding proteins of HMEC cells cultured in Normoxic or Hypoxic conditions for 24 hours. (N of 3). Data shows relative ratio of RNAs compared to 28S rRNA

Protein levels of the enzymatically active subunit of the methyltransferase, Mettl3, were decreased in immortalized and transformed cells, but Mettl14 expression was increased (Figure 3.5). In addition, ZNF217, which is known to sequester Mettl3 and inhibit its methyltransferase activity [16], is also increased in the immortalized and transformed cells. Also in agreement with the decreased m6A levels, the demethylases ALKBH5 and FTO were both increased in the immortalized and transformed cells. This data suggested that the loss of m6A observed in the transformed cells is due to a loss of the methyltransferase, Mettl3, and the increase of the demethylases, ALKBH5 and FTO.

The m6A-recognizing RNA binding proteins YTHDF1, YTHDF2 and YTHDC1 were also altered in the immortalized and transformed cells lines. YTHDF1 levels were increased, but YTHDF2 levels were decreased in the immortalized and transformed cells. The blots for YTHDC1 interestingly contained a shift from a lower molecular weight (the correct expected molecular weight) to a much higher molecular weight (the observed molecular weight given in the details of the antibody). The switch in protein levels from YTHDF2 to YTHDF1 suggests that the primary outcome of the m6A function is no longer degradation of the mRNA, but rather increased translational efficiency. Unfortunately, the observed protein levels of the methyltransferase, demethylases or RNA binding proteins were unable to explain the increase in m6A levels in hypoxia suggesting that the m6A increase in hypoxia must be due to some other process.

Differential methylation in specific mRNAs

Protein levels of m6A methyltransferases and demethylases could not explain why m6A is increased in hypoxia in the immortalized and transformed HMECs. Two potential

Figure 3.5

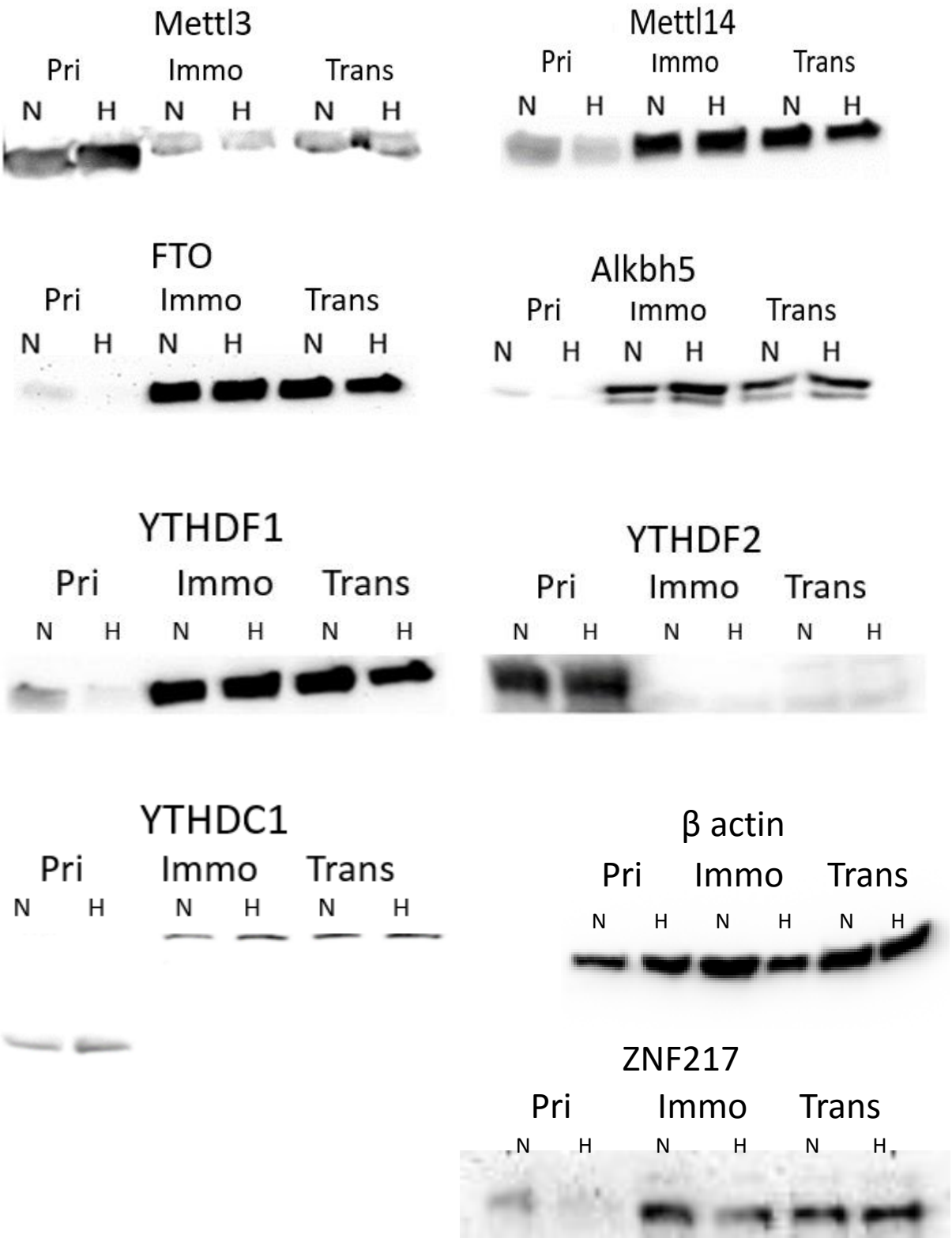

Mettl3
Pri
Immo
Trans
N H N H N H
Mettl14
Pri
Immo
Trans
N H N H N H
FTO
Pri
Immo
Trans
N H N H N H
Alkbh5
Pri
Immo
Trans
N H N H N H
YTHDF1
Pri
Immo
Trans
N H N H N H
YTHDF2
Pri
Immo
Trans
N H N H N H
YTHDC1
Pri
Immo
Trans
N H N H N H
β actin
Pri
Immo
Trans
N H N H N H
ZNF217
Pri
Immo
Trans
N H N H N H

Figure 3.5: Protein levels of m6A methyltransferases, demethylases and RNA binding proteins.

Western blots of 50 µg of protein lysates of normoxic (N) or hypoxic (H) primary (pri), immortalized (immo), and oncogenically transformed (trans) HMEC cells. (Representative of 3 experiments).

explanations could explain the increase of m6A in hypoxic cells. We have recently reported that the m6A modification stabilizes mRNA in hypoxic conditions in HEK293T cells [11]. Therefore, it is possible that the increase of m6A is simply due to a build-up of stabilized methylated mRNA. Secondly, it is possible that methylation rates have increased in newly transcribed RNA in hypoxic conditions. In order to test this second possibility m6A RNA immunoprecipitation (MeRIP) as described previously [11] was used to initially identify specific mRNA targets that increased m6A levels after 24 hours of hypoxic conditions. Not surprisingly, many of the same targets identified in our previous report using HEK-293T cells, including Glut1, Jun, VHL, and Dusp1 also increased m6A levels in the oncogenically transformed HMEC line (Figure 3.6 blue bars). MeRIP of the immortalized cells showed a similar trend as seen in the transformed cells while no significant differences in m6A levels in these same targets were observed in the Primary cells (Figure 3.7). This data demonstrates an increase in the m6A content of these mRNAs from transformed HMECs in response to hypoxic exposure.

Additionally, the increase of m6A in specific mRNA targets in hypoxic conditions were compared to m6A in newly transcribed RNAs. In order to measure methylation rates of newly transcribed RNA in hypoxia, a uridine analog, 4-thiouridine (4sU), was added to the culture media one hour until harvest to be incorporated into all newly transcribed RNA. This newly transcribed RNA was then biotinylated and separated from old (unlabeled) mRNA using streptavidin beads. After elution of the newly transcribed RNA from the streptavidin beads, MeRIP was then used to pull down m6A methylated RNA from this population of new RNA. Interestingly, newly transcribed Glut1, Jun, VHL,

Figure 3.6

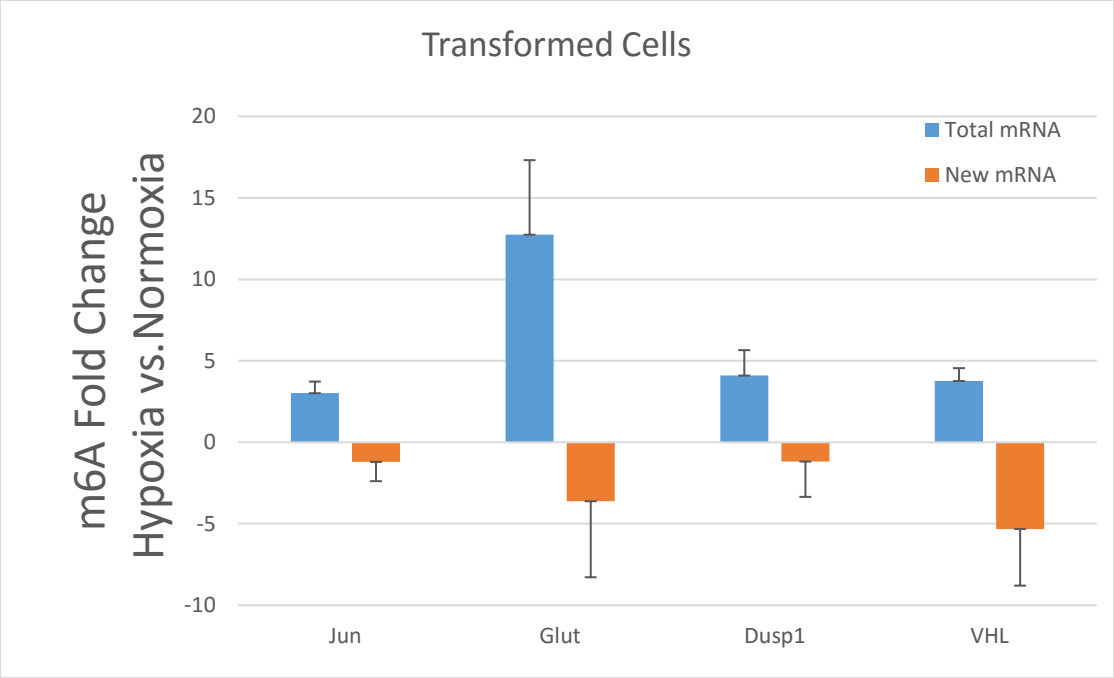
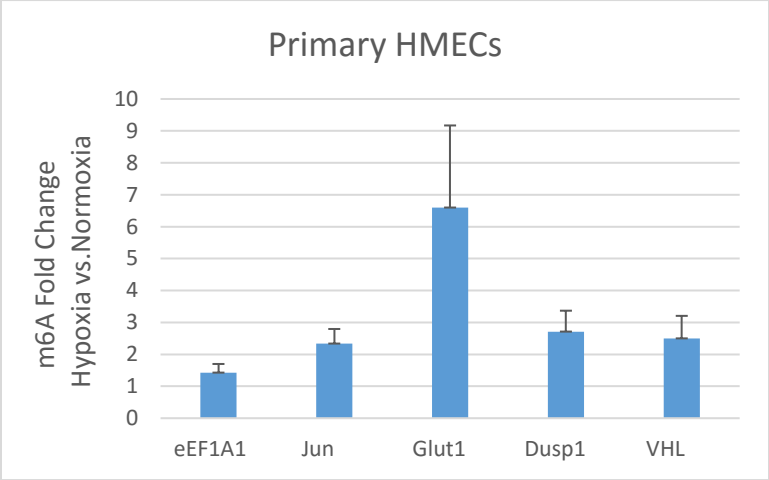

Transformed Cells
m6A Fold Change
Hypoxia vs.Normoxia
20
15
10
5
0
-5
-10
Total mRNA
New mRNA
Jun
Glut
Dusp1
VHL

Figure 3.6: m6A methylation rates are decreased in hypoxic transformed HMEC cells.

(Blue Bars) MeRIP of 100 ng of mRNA from oncogenically transformed cells or (Red Bars) 100 ng of newly transcribed RNA isolated via 4-thiouracil labelling, biotinylation and streptavidin pull downs. Cells were grown in Normoxic or Hypoxic conditions for 24 hours and RNA was quantified by qRT-PCR. Fold enrichments calculated from immunoprecipitated mRNA levels normalized to input RNA and expressed as a ratio of hypoxia/normoxia. *P ≤ 0.05 by Paired Student's t-test. Error bars represent SEM of 4 experiments.

Figure 3.7

A.


Primary HMECs
m6A Fold Change Hypoxia vs.Normoxia
10
9
8
7
6
5
4
3
2
1
0
eEF1A1
Jun
Glut1
Dusp1
VHL

B.

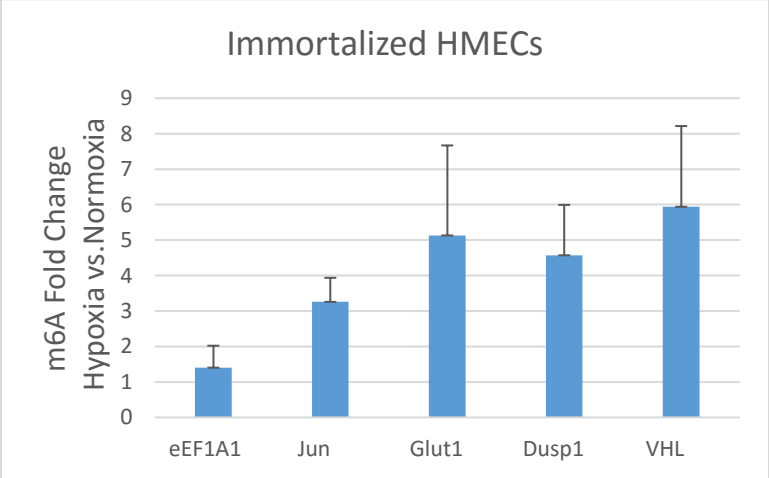

Immortalized HMECs
m6A Fold Change Hypoxia vs.Normoxia
9
8
7
6
5
4
3
2
1
0
eEF1A1
Jun
Glut1
Dusp1
VHL

Figure 3.7: m6A IP in Primary and Immortalized HMECs MeRIP of 100 ng of mRNA from HMEC primary (A) or immortalized (B) cells grown in Normoxic or Hypoxic conditions for 24 hours quantified by qRT-PCR. Fold enrichments calculated from immunoprecipitated mRNA levels normalized to input mRNA and expressed as a ratio of hypoxia/normoxia. *P ≤ 0.05 by Paired Student's t-test. Error bars represent SEM of 3-4 experiments.

and Dusp1 mRNA had a decreasing trend of m6A methylation (Figure 3.6 red bars), suggesting that methylation rates of these newly transcribed RNAs were not increasing. This data then also suggests that the increase in m6A in hypoxic conditions was not due to a change in the methylation rates of newly transcribed mRNA, but rather a buildup of stabilized mRNAs.

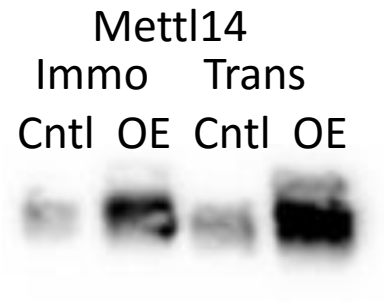
HIF controls hypoxic m6A levels in specific targets

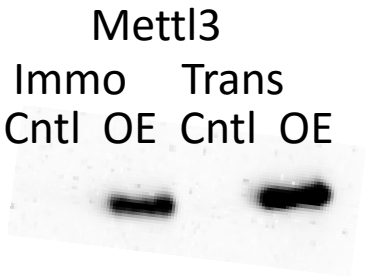
To further investigate how m6A is increased under hypoxia, we wanted to determine if Hypoxia Inducible Factor (HIF) was involved. HIF-1α and HIF-2α were knocked down via siRNA in oncogenically transformed cells which were then exposed to hypoxia. Knockdown of HIF-1α and HIF-2α was confirmed via western blot analysis (Figure 3.8). m6A content in individual mRNAs were measured once again by MeRIP followed by qPCR. Knockdown of HIF-1α and HIF-2α prevented the hypoxic increase in m6A levels in many of our specific targets including Glut1, VHL, and Dusp1 (Figure 3.9). This suggests that the increase in m6A in hypoxia is at least partially due to the hypoxic induction of HIF.

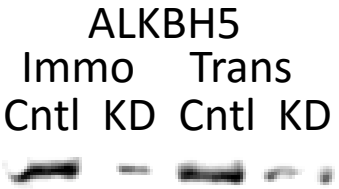
Phenotypic effects of m6A on breast cells

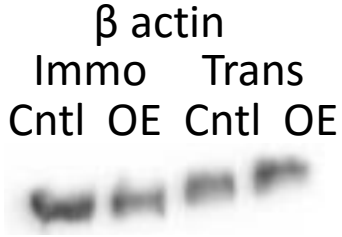
The goal of this project was not only to understand the differences in m6A levels between the stages of a genetically defined breast cancer model, but also to understand how those changes affect the cells phenotypically. Because the immortalized and oncogenically transformed cell lines contained decreased m6A levels, it was initially hypothesized that increasing m6A content in these cells might lead to a primary-like cell state. Surprisingly, as shown in figure 3.10 A-B, proliferation of the transformed cell

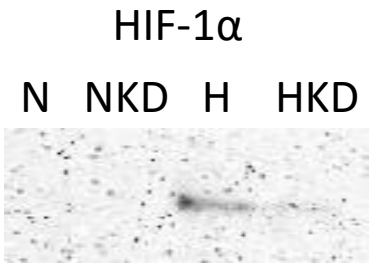
Figure 3.8


Mettl14
Immo
Trans
Cntl
OE
Cntl
OE


Mettl3
Immo
Trans
Cntl
OE
Cntl
OE


ALKBH5
Immo
Trans
Cntl
KD
Cntl
KD


β actin
Immo
Trans
Cntl
OE
Cntl
OE


HIF-1α
N
NKD
H
HKD

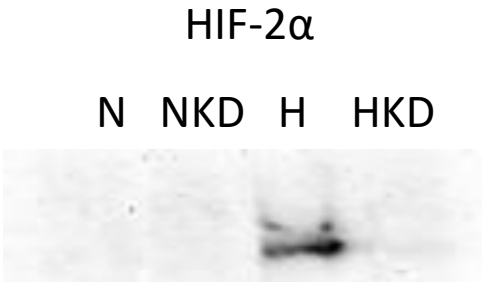

HIF-2α
N
NKD
H
HKD

Figure 3.8 Protein levels of Mettl3/14 after overexpression, Alkbh5 after Knockdown, and HIF-1 and 2α after knockdown.

Western blots of 50 µg of protein lysates of control (Cntl), Overexpression (OE) or knockdown (KD) of immortalized (immo), and oncogenically transformed (trans) cells. HIF KD blots compare normoxia (N) with or without knockdown (NKD and hypoxia (H) with or without knockdown (HKD).

Figure 3.9

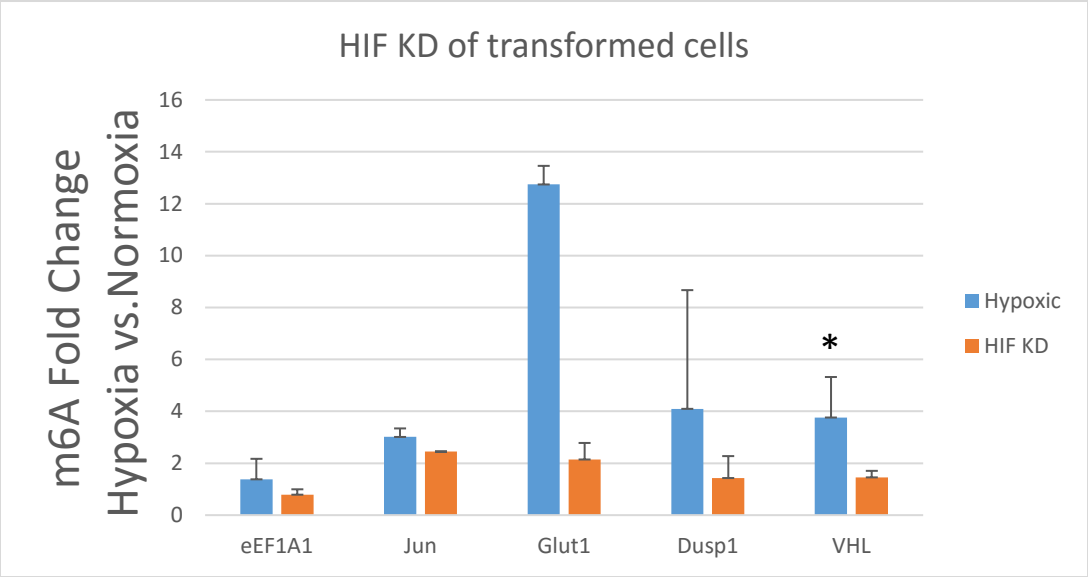
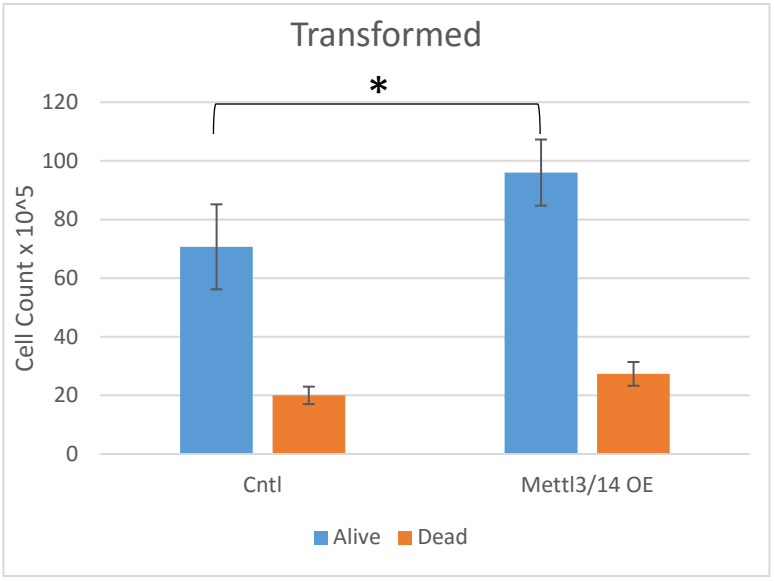

HIF KD of transformed cells
m6A Fold Change
Hypoxia vs.Normoxia
16
14
12
10
8
6
4
2
0
eEF1A1
Jun
Glut1
Dusp1
VHL
*
Hypoxic
HIF KD

Figure 3.9: Hypoxic increases in m6A are HIF dependent.

MeRIP of 100 ng of mRNA from oncogenically transformed cells. Cells were cultured with or without HIF-1α and HIF-2α 72 hour knockdown and either Normoxic or Hypoxic conditions for 24 hours. RNA was quantified by qRT-PCR. Fold enrichments calculated from immunoprecipitated mRNA levels normalized to input RNA and expressed as a ratio of hypoxia/normoxia. For statistical analysis, ratios of hypoxia/normoxia were compared between control and HIF knockdown samples. *P ≤ 0.05 by Paired Student's t-test. Error bars represent SEM of 4 experiments.

Figure 3.10

A.



B.

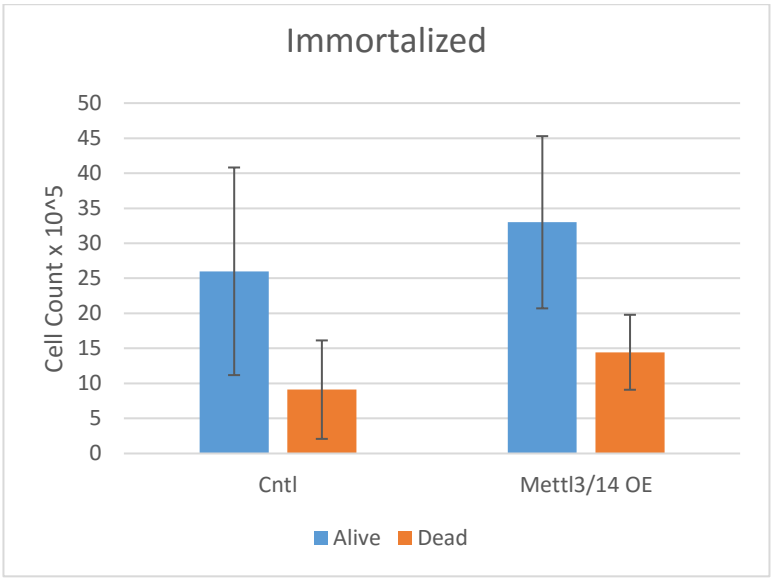


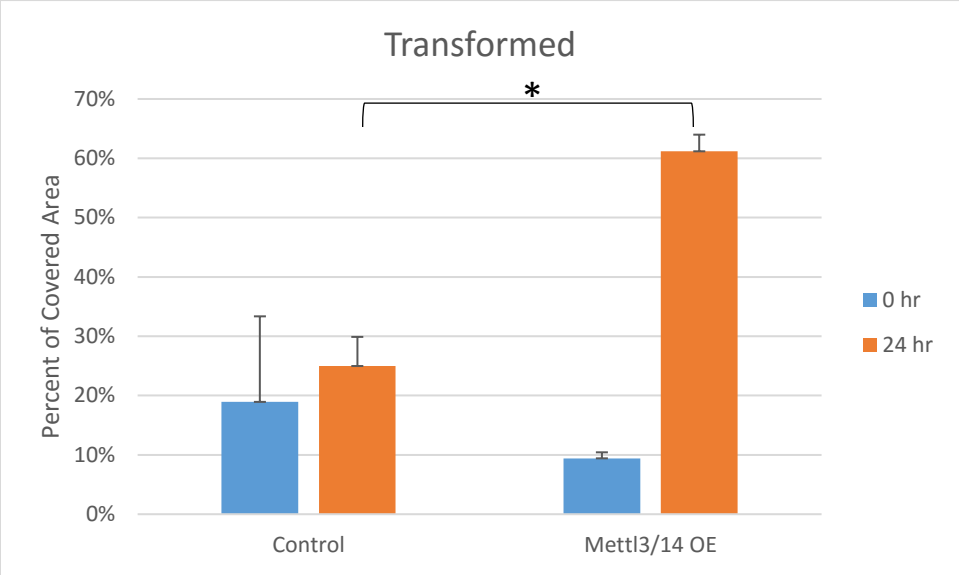
Figure 3.10: Mettl3 and 14 Overexpression leads to increased proliferation of oncogenically transformed HMECs

(A) Mettl3/14 overexpression (OE) significantly increased cell proliferation in oncogenically transformed HMECs cells when compared to negative plasmid transfection (cntl). (N of 3) (B) Mettl3/14 overexpression did not significantly increase cell proliferation in immortalized HMEC cells, however, there is an increasing trend of proliferation (N of 3).

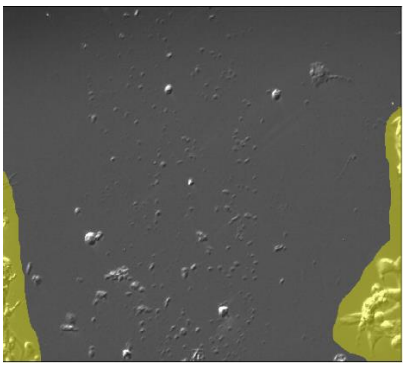
lines was significantly increased after Mettl3 and Mettl14 overexpression, however, the immortalized cells were not affected. Additionally, wound healing scratch assays showed that Mettl3 and 14 overexpression aided the transformed cells in wound healing, however very little differences were seen in immortalized cells when compared to controls (Figure 3.11 A-E and 3.12 A-E). In addition, a trend showing increased wound healing via knockdown of the demethylase ALKBH5 in transformed cells, but not in immortalized cells, suggests that this effect is due to increased m6A levels rather than increased Mettl3/14 levels (Figure 3.13A-E, 3.14A-E). Confirmation of Mettl3/14 overexpression and Alkbh5 knockdown is shown in Figure 3.8. Furthermore, upon Mettl3 and 14 overexpression, transformed cells, but not immortalized cells (data not shown) showed increased invasion through matrigel (Figure 3.15 A-E). These phenotypic assays suggest that increased m6A in the transformed cells did not revert these cells to a more primary-like cell state but instead increased their metastatic potential. Remarkably, in terms of wound healing, increased m6A actually mimicked the response to hypoxic conditions (Figure 3.16 A-E). Hypoxia led to increased m6A in the transformed cells (Figure 3.2), and increased wound healing (Figure 3.16A-E), but had little effect on wound healing in the immortalized cells (Figure 3.17A-E). Overall, this suggests that m6A may not play a role in cellular transformation, but rather aids and fine-tunes current processes in the cell to overcome stress and promote cellular growth and survival.

Figure 3.11

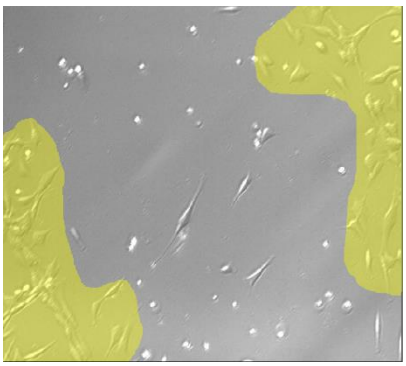
A.



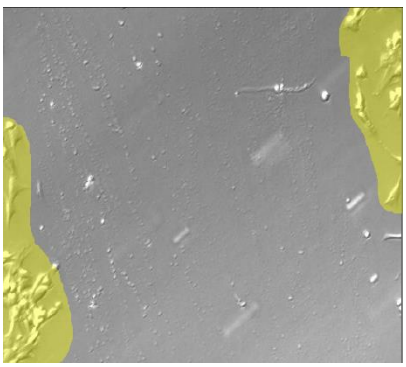
B. Cntrl 0 hr



C. Cntrl 24 hr



D. Mettl3/14 OE 0 hr



E. Mettl3/14 OE 24 hr

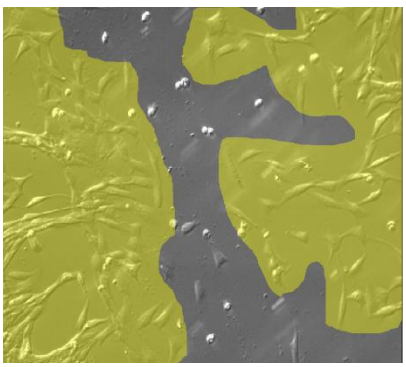


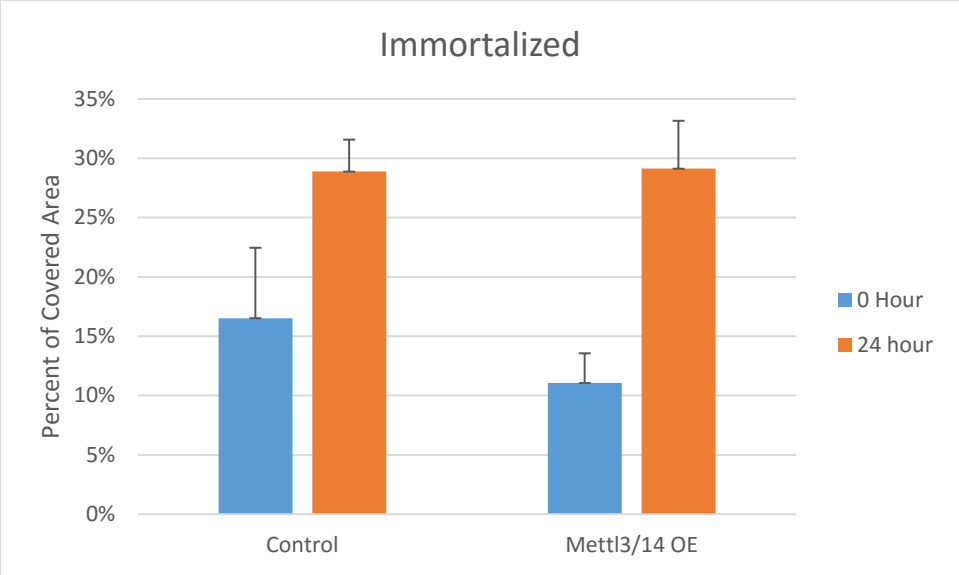
Figure 3.11: Mettl3/14 Overexpression increases wound healing in oncogenically transformed HMECs

(A) Mettl3/14 overexpression (OE) significantly increased wound healing after 24 hours in oncogenically transformed HMECs cells when compared to negative plasmid transfection (control). (N of 3)

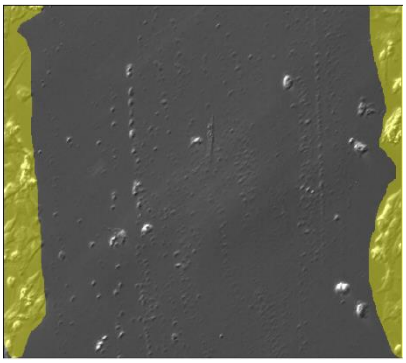
(B-E) Images of scratch assays of negative plasmid transfection (Control) and Mettl3/14 Overexpression (OE) at 0 and 24 hour time points. (Images representative of 3 experiments).

Figure 3.12

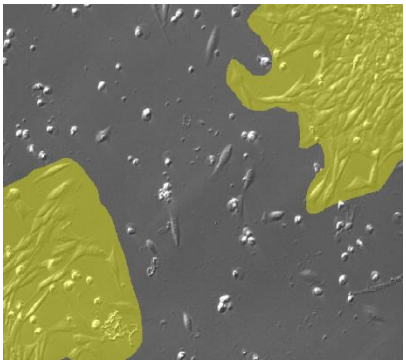
A.



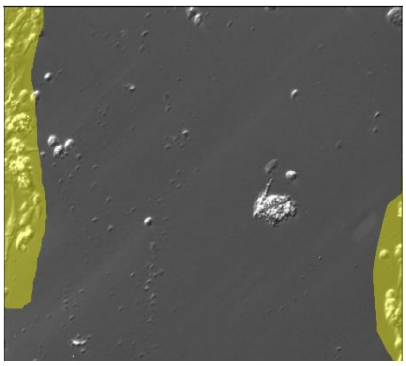
B. Cntrl 0 hr



C. Cntrl 24 hr



D. Mettl3/14 OE 0 hr



E. Mettl3/14 OE 24 hr

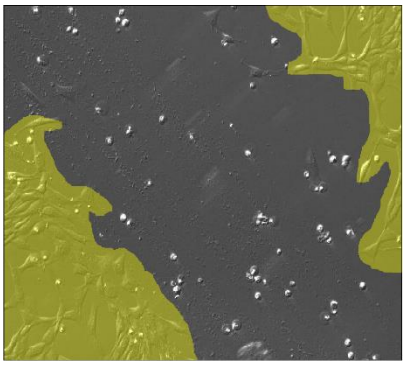


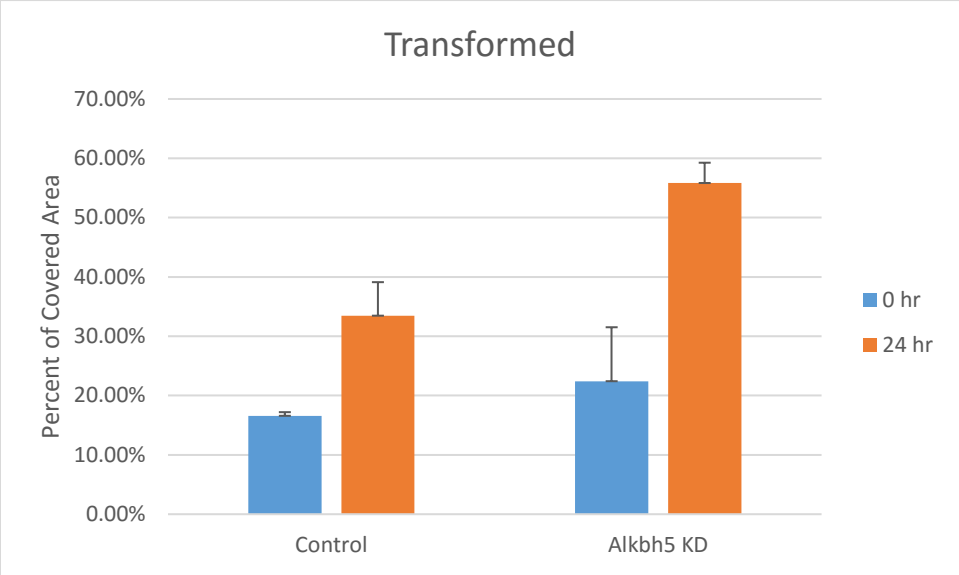
Figure 3.12: Mettl3/14 Overexpression does not increase wound healing in immortalized HMECs

(A) Mettl3/14 overexpression (OE) had little effect on wound healing after 24 hours in immortalized HMECs cells when compared to negative plasmid transfection (control). (N of 3)

(B-E) Images of scratch assays of negative plasmid transfection (Control) and Mettl3/14 Overexpression (OE) at 0 and 24 hour time points. (Images representative of 3 experiments).

Figure 3.13

A.



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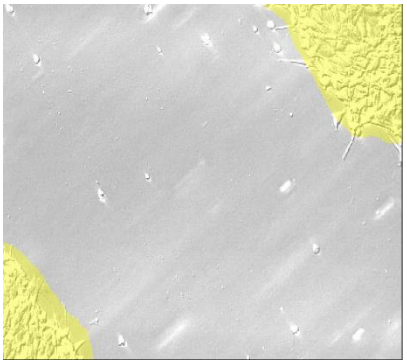
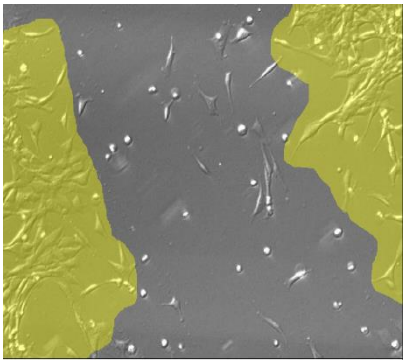
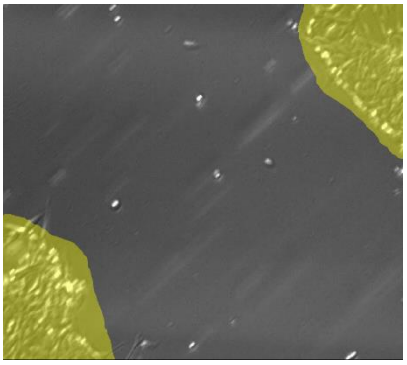


C. Cntrl 24 hr



D. Alkbh5 KD 0 hr



E. Alkbh5 KD 24 hr

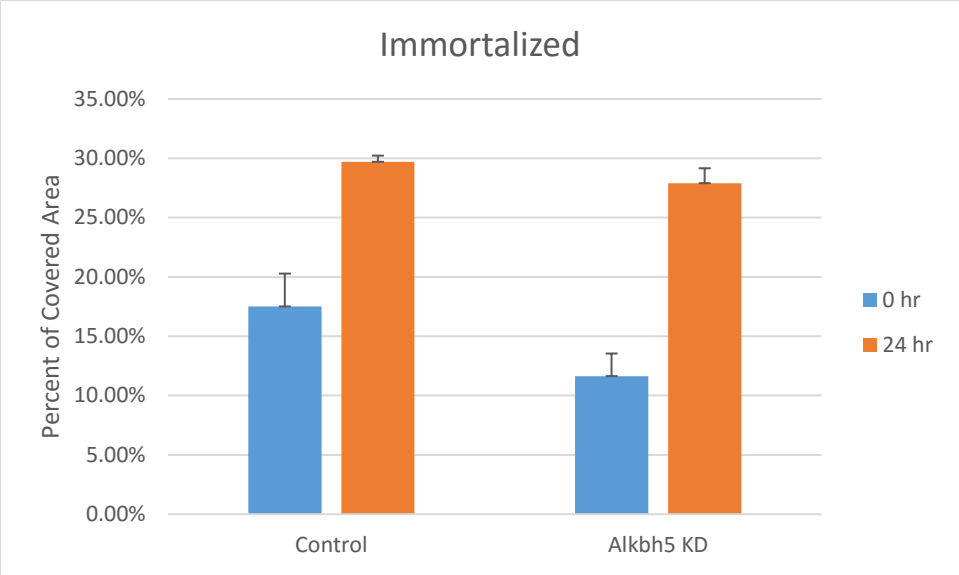
Figure 3.13: Demethylase ALKBH5 knockdown increases wound healing in Oncogenically Transformed HMECs.

(A) Alkbh5 Knockdown (KD) increased wound healing after 24 hours in oncogenically transformed HMECs cells when compared to negative plasmid transfection (control). (N of 3)

(B-E) Images of scratch assays of negative plasmid transfection (Control) and Alkbh5 KD at 0 and 24 hour time points. (Images representative of 3 experiments).

Figure 3.14

A.



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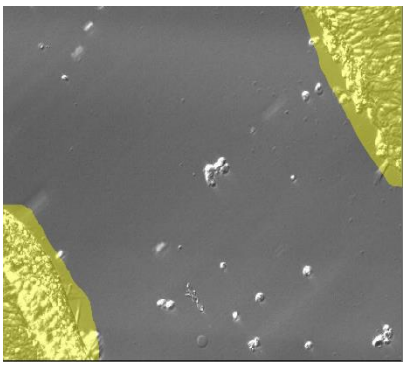

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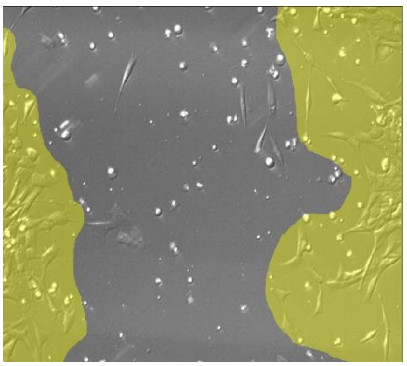
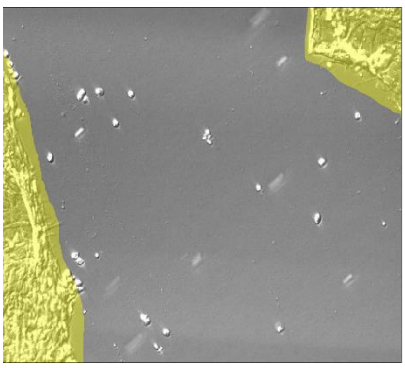


D. Alkbh5 KD 0 hr



E. Alkbh5 KD 24 hr

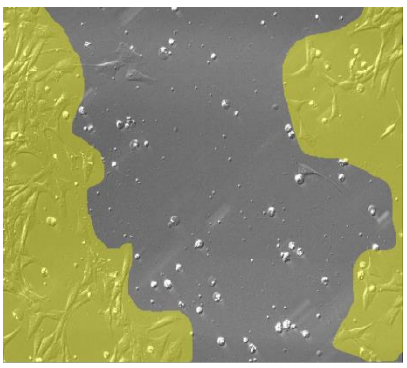


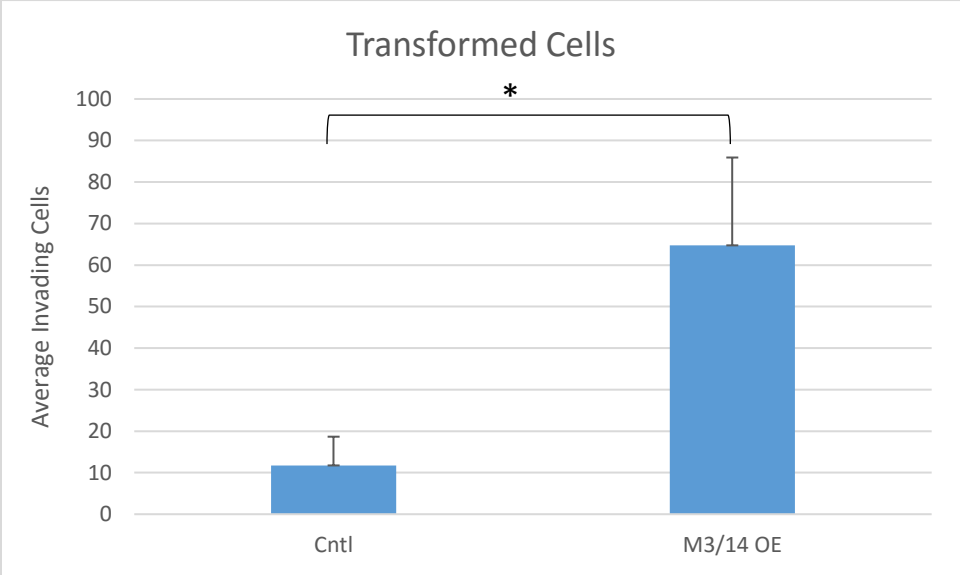
Figure 3.14: Demethylase ALKBH5 knockdown does not increase wound healing in immortalized HMECs

(A) Alkbh5 knockdown (KD) had little effect on wound healing after 24 hours in immortalized HMECs cells when compared to negative plasmid transfection (control). (N of 3)

(B-E) Images of scratch assays of negative plasmid transfection (Control) and Alkbh5 knockdown at 0 and 24 hour time points. (Images representative of 3 experiments).

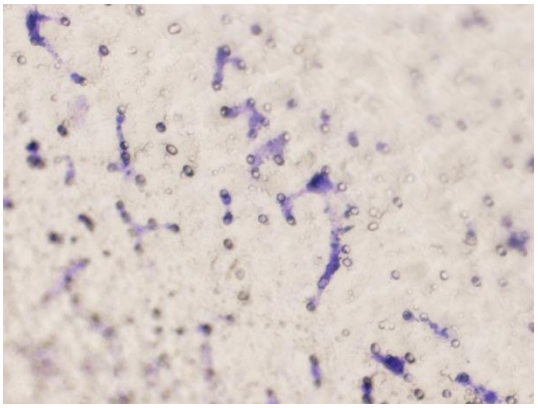
Figure 3.15

A.



B.

Control



Mettl3/14 Overexpression

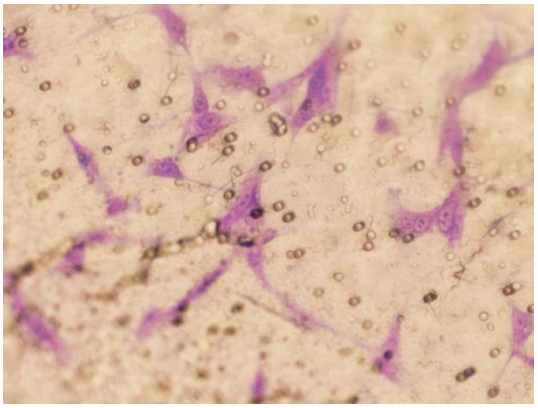


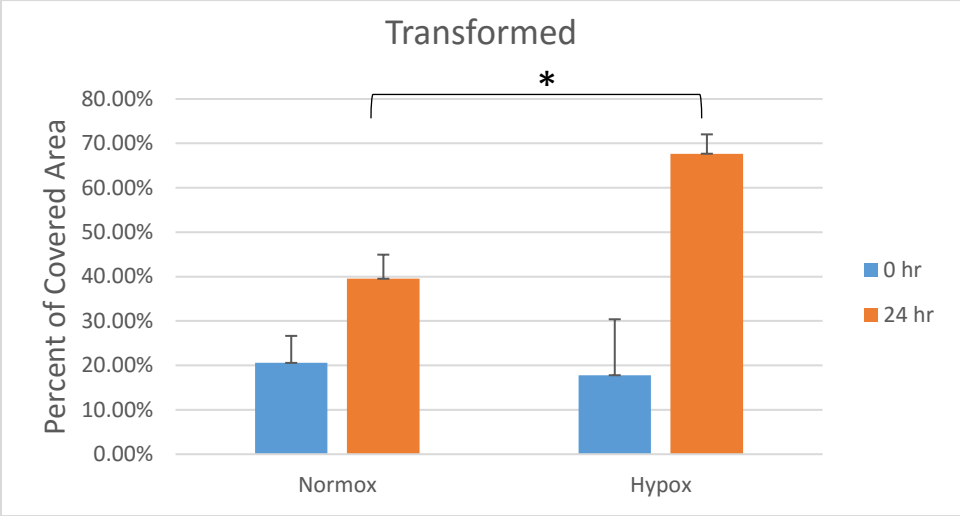
Figure 3.15: Mettl3/14 Overexpression increases invasion in oncogenically transformed HMECs

(A) Mettl3/14 overexpression (OE) significantly increased invasion of oncogenically transformed HMECs cells when compared to negative plasmid transfection (control). (N of 4). Average invading cells shows number of cells that crossed the matrigel.

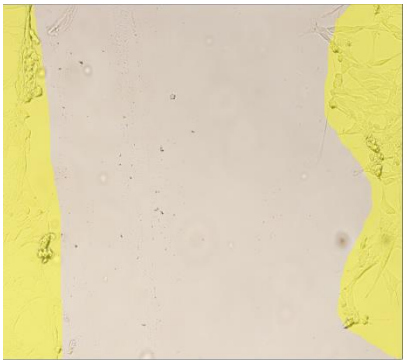
(B) Images of membrane from matrigel invasion assays of negative plasmid transfection (Control) and Mettl3/14 Overexpression. (Images representative of 4 experiments).

Figure 3.16

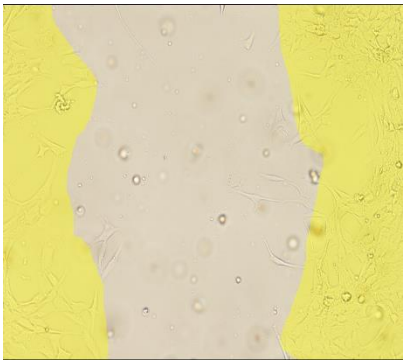
A.



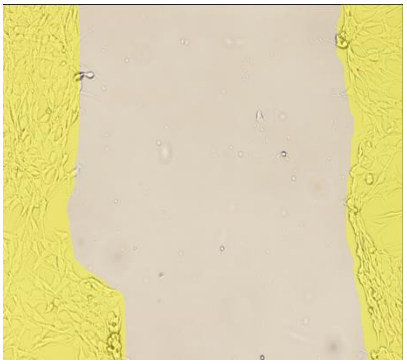
B. Normox 0 hr



C. Normox 24 hr



D. Hypox 0 hr



E. Hypox 24 hr

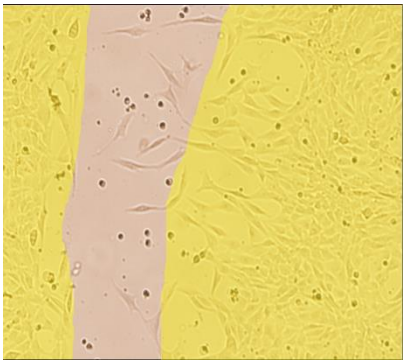


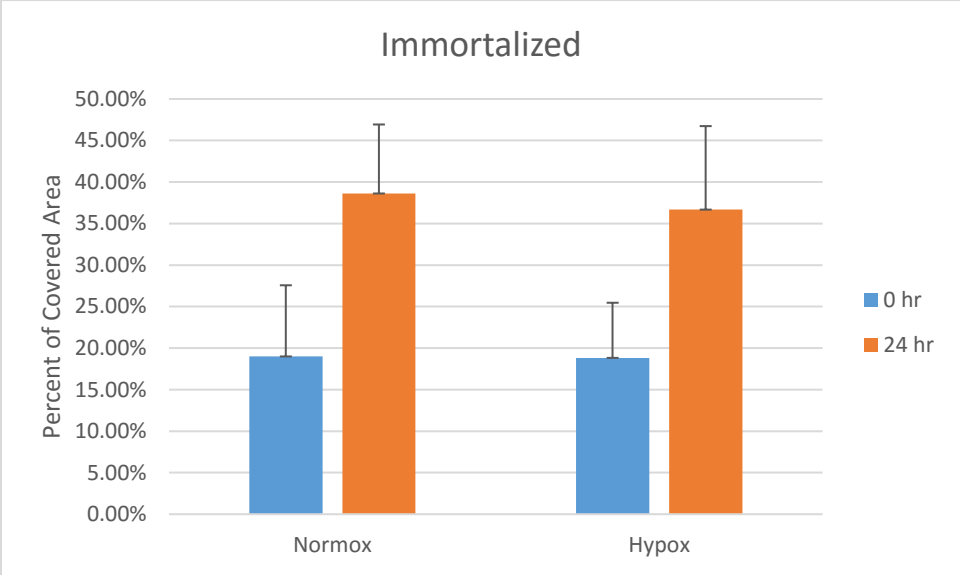
Figure 3.16: Hypoxia increases wound healing in oncogenically transformed HMECs

(A) Hypoxia (Hypox) significantly increased wound healing after 24 hours in oncogenically transformed HMECs cells when compared to normoxia (Normox). (N of 3)

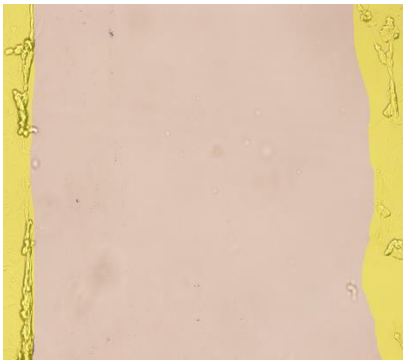
(B-E) Images of scratch assays of normoxic and hypoxic conditions at 0 and 24 hour time points. (Images representative of 3 experiments).

Figure 3.17

A.

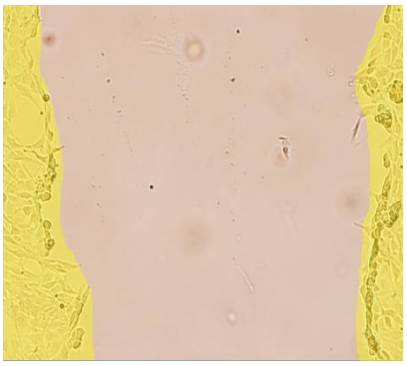


B. Normox 0 hr



C. Normox 24 hr

D. Hypox 0 hr



E. Hypox 24 hr

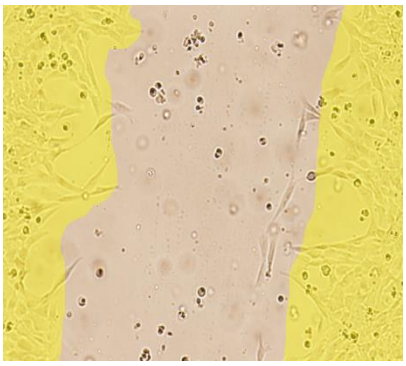


Figure 3.17: Hypoxia does not increases wound healing in immortalized HMECs

(A) Hypoxia (Hypox) had little effect on wound healing after 24 hours in immortalized HMECs cells when compared to Normoxia (Normox). (N of 3)

(B-E) Images of scratch assays of normoxic and hypoxic conditions at 0 and 24 hour time points. (Images representative of 3 experiments).

Summary

These findings indicate that m6A methylation levels can be altered in human breast cancer cells when compared with their normal primary cell countertype. Specifically, in this model, m6A decreases upon immortalization, and further decreases upon oncogenic transformation of human mammary epithelial cells. Additionally, consistent with our previous findings, hypoxia increases mRNA m6A levels in transformed cell types [11]. Ultimately, these increases in m6A in hypoxia are HIF regulated, and are not due to increased protein levels of the methyltransferase or methylation rates. Interestingly, increasing m6A methylation by overexpressing the methyltransferases and/or hypoxic exposure in the transformed cells led to further developed cancer-like phenotypes indicating that differing stages of cells may utilize m6A in different ways in order to increase their own chances for survival and growth.

Discussion

Although gene expression in breast cancer has been extensively studied, the role of RNA modifications in breast cancer is not well known. Studies involving these RNA modifications, including the m6A modification, may lead to a better understanding of gene regulation in breast cancers and potentially other cancers as well. This report utilizes a genetically defined progressive breast cancer model which is advantageous in studying gene regulation because any phenotypic changes may be mapped back to a specific genetic alteration.

In this study, LC-MS/MS results indicate that the m6A modification decreases after immortalization and oncogenic transformation of primary HMECs. Initially, these

data seemed to suggest that the loss of the m6A modification may lead to a more oncogenic phenotype. However, hypoxia, a cellular stress condition involved in increasing tumorigenic phenotypes, drives the m6A modification back to near normal primary cell levels. Previous reports have shown total RNA m6A levels in breast cancer stem cells decrease in hypoxia conditions through HIF mediated induction of the demethylase ALKBH5 and Mettl3 sequestration by ZNF217 [15, 88]. These reports indicated that hypoxia induces the breast cancer stem cell phenotype through the decrease in m6A. The results seen here differ from these previous reports possibly due to differences in model systems. For example, these previous reports show that m6A levels in specific mRNA, including Nanog, is decreased in hypoxia. However, Nanog is not highly expressed in our cell lines so no differences in m6A were observed (data not shown). In addition, these previous results also used total RNA rather than mRNA to measure differences in global m6A levels.

To understand why m6A levels are increased in hypoxic conditions in our model system in the immortalized and oncogenically transformed cells, protein expression of the methyltransferase, demethylases, and m6A RNA binding proteins were measured. Interestingly, the enzymatically active subunit of the methyltransferase complex, Mettl3, is decreased in the immortalized and transformed cells, and ZNF217 which sequesters Mettl3 is increased. Additionally, the two known m6A demethylases, ALKBH5 and FTO, increase in the immortalized and transformed cells. Therefore, it can be concluded that the decrease in m6A methylation in the immortalized and transformed cells is due to a loss in methylation potential and an increase in demethylation potential. In addition, protein expression of m6A specific RNA binding proteins also change in the

immortalized and transformed cells. YTHDF2, an m6A RNA binding protein which leads to degradation of the methylated RNA [9, 40], decreased in the immortalized and transformed cells. In contrast, YTHDF1, another m6A specific RNA binding protein involved in methylated mRNA translational efficiency increases in the immortalized and transformed cells [10]. These contrasting changes in RNA binding proteins suggest that the remaining methylated RNA have increased translational efficiency through increased YTHDF1 levels and an increase in stability due to a decrease in YTHDF2 levels. The RNA binding protein YTHDC1 interestingly appears expressed at a higher molecular weight in the immortalized and transformed cells. This may possibly be due to dimerization of YTHDC1, however this has not been confirmed. It would also not explain why YTHDC1 would dimerize in certain cell types but not in others. It was also observed that hypoxia does not lead to changes in any of the aforementioned proteins suggesting that the increase of m6A in this stress condition cannot be explained by through protein expression.

Because the increase of m6A in hypoxia cannot be explained by protein levels of methyltransferases and demethylases, alternative explanations including methylation rates of newly transcribed RNAs were investigated. We previously reported that increased m6A methylation in hypoxia leads to stabilization of mRNAs [11]. Therefore it is possible that the increase in m6A methylation is due to stabilization and accumulation of methylated mRNAs. However, in order to test if methylation rates increased in hypoxic conditions, newly transcribed RNAs were pulse-labeled, isolated and immunoprecipitated using an m6A antibody. These experiments showed that methylation rates in newly transcribed RNA were not increased, and in fact were

trending towards a decrease in specific targets where m6A levels were initially increased. Therefore, it seems likely that the increase in m6A levels is simply due to the accumulation of stabilized mRNA. However, there are a few other potential but unlikely possibilities that we have yet to investigate. It is possible that methylation is occurring to some degree within the cytoplasm in order to methylate mature mRNA, or that decreased demethylation is occurring on cytoplasmic mRNA. A recent study however, has shown that m6A methylation is not dynamic within the cytoplasmic, which would suggest that neither methylation nor demethylation levels are altered [21].

Because the hypoxic response through HIF is crucial for survival and tumorigenesis of cancer cells under hypoxic conditions, the effect of HIF on m6A levels in hypoxia was measured. Previous reports once again contradict these results showing that in breast cancer stem cells, hypoxic activation of HIF led to decreased RNA methylation through HIF mediated induction of the demethylase ALKBH5 and Mettl3 suppressor ZNF217 [15, 88]. However, because in our model system there is an increase in m6A in hypoxia rather than a decrease, HIF may be regulating these changes in m6A as well. Indeed, knockdown of HIF decreased m6A levels in many of our specific targets including Glut1, Dusp1, and VHL suggesting that HIF is responsible for the increase in m6A in these targets. The conflicting data with the previous report may be explained through a difference in model system, and highlights the importance of understanding the m6A modification in all breast cancer systems.

Understanding mechanisms behind m6A methylation is important in breast cancer, as it may lead to a better understanding of the cancer itself. However, it is also important to observe the phenotypic effects that m6A has directly on breast cancer. As

stated previously, immortalization and oncogenic transformation of HMEC cells led to a decrease in m6A methylation. Initially, it seemed possible that an increase of m6A in these transformed cells would drive cells towards a more primary phenotype. However, m6A was increased after 24 hours of hypoxia, a condition known to promote tumorigenesis. Ultimately, when Mettl3 and Mettl14 were overexpressed thereby increasing m6A levels, the oncogenically transformed cells exhibited increased proliferation, wound healing, and invasion. Increased wound healing was also seen in these same cells under hypoxia. Through these results, it appears likely that an increase in m6A either through methyltransferase overexpression or a HIF mediated increase in hypoxia leads to a more cancerous phenotype. Interestingly, the immortalized cells did not show the same effect as the oncogenically transformed, suggesting that the increased m6A was working in tandem with active expression of the Ras pathway in order to alter these phenotypes. While we cannot completely rule out the possibility that the overexpression of Mettl3 and Mettl14 itself had an effect on these phenotypes, it seems likely that the phenotypic changes are m6A dependent as knockdown of the demethylase ALKBH5 had similar effects to overexpression of Mettl3 and Mettl14.

In conclusion, this study demonstrates that m6A methylation is important for the phenotypic progression of breast cancer. Hypoxia plays a role in this progression through increases m6A levels in breast cancer through HIF activation. This hypoxic response can be mimicked in normal cells by overexpressing the methyltransferase subunits Mettl3 and Mettl14.

Materials and Methods

Cell Lines

HMEC Primary cell lines cells were obtained directly from Lonza (Walkersville, MD) and maintained in Mammary Epithelial Basal Medium (MEBM) (Lonza) and supplemented with Mammary Epithelial Cell Growth Medium (MEGM) BulletKit (Lonza) along with 2mM Glutamine (Corning/Mediatech), and 1X Pen/Strep (Corning/Mediatech) and passaged when approximately 85-90% confluent. Cells were tested for mycoplasma upon receipt. For experiments, cells were plated on 10cm dishes (CytoOne, USA Scientific, Orlando, FL) allowed to attach/recover for 18-24 hours. The next day, the media was removed and replaced with fresh media. Hypoxic treatments were carried out in a Ruskin In Vivo 400 Hypoxia Hood (The Baker Company, Sanford, ME) maintained at 37°C, 5% CO_2, 70% humidity and 1% oxygen. All other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

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). For RNA extraction from ribonucleoprotein immunoprecipitations (RNP-IP) and sucrose gradients, GlycoBlue (Life Technologies) was added as a carrier during the precipitation step. RNA quality and quantity was determined via NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA).

PolyA$^+$ RNA purification

PolyA$^+$ 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.

LC-MS/MS of PolyA$^+$ RNA

Purified PolyA$^+$ RNA was digested to individual nucleosides and modified nucleosides were quantified as previously described [36]. Briefly, digestion was performed with nuclease P1 (Sigma, 2U) in buffer containing 25 mM NaCl and 2.5 mM ZnCl2 for 2h at 37ºC, followed by incubation with Antarctic Phosphatase (NEB, 5U) for an additional 2h at 37ºC. Nucleosides were then separated and quantified at the Duke Molecular Physiology Institute using UPLC-MS/MS as previously described [152], except acetic acid replaced formic acid in the mobile phase.

Western Blots

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 (30-50 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). Transfer was verified via Ponceau S staining then 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), anit-rabbit (GE Healthcare), or Rabbit anti-goat (Novus Biologicals, Littleton, CO), washed again with TBST, detected using Bio-Rad Clarity Western ECL Substrate (Bio-Rad Laboratories), and imaged via MYECL Imager (Thermo Scientific).

Assaying newly transcribed mRNA

Newly Transcribed mRNAs were isolated following pulse-labelling with 4-thiouracil. Cells were treated with 200 µM 4SU (Sigma-Aldrich) for 1 hour. RNA isolated via Trizol was biotinylated by labeling 50 µg RNA in a reaction mixture with 50 µl 10x Tris/EDTA buffer (TE), 100 µl 1mg/ml Biotin-HPDP (EZ-Link Biotin HPDP, Thermo Scientific, Waltham, MA) in dimethylformamide (DMF), and RNase free H_2O brought to 400 µl. Mixture was incubated in the dark with rotation for 1.5 hours. Biotinylated RNA was extracted using an equal volume of chloroform/isoamyl alcohol (24:1) 2x in phase lock gel heavy tubes (5 Prime, Gaithersburg, MD) followed by RNA precipitation with isopropanol. RNA was heated to 65ºC for ten minutes and placed immediately on ice. RNA was added to Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific) that had been thoroughly washed and resuspended in 2X streptavidin binding buffer (2X TE, 2M NaCl). The RNA bead mixture was incubated with rotation for 30 minutes. Beads were washed 5X with 65ºC Wash Buffer (1XTE, 1M NaCl, 0.1% Tween20) and the supernatant was kept containing the non-labeled RNA. Three rounds of 100 mM dithiothreitol (DTT) elution followed by 1 round of TE eluted labeled RNA from the beads. RNA was isolated via Isopropanol and resuspended in 10 µl of water.

m6A mRNA Immunoprecipitation (MeRIP)

m6A Ribonucleoprotein Immunoprecipitation reactions were performed by first isolating PolyA$^+$ RNA from normoxic and hypoxic cells. Protein G Dynabeads (Thermo Fisher Scientific, Baltics UAB) were washed 3X in 1 mL of IPP buffer (10mM Tris-HCL pH7.4, 150mM NaCl, 0.1% NP-40). 25 µl of beads required per IP. Anti-N6-methyladenosine mouse monoclonal antibody (EMD Millipore, Temecula, CA, MABE1006) was added to the beads (5 µg/IP) and brought up to 1mL with IPP buffer. Bead mixture was tumbled for 16 hours at 4ºC. Beads were washed 5X with IPP buffer and 100ng of PolyA$^+$ RNA was added to the beads along with 1mM DTT and RNase out. The mixture was brought up to 500 µl with IPP buffer. Bead mixture was tumbled at 4ºC for 4 hours. Beads were washed 2X in IPP buffer, placed into a fresh tube, and washed 3X more in IPP buffer. m6A RNA was eluted off the beads by tumbling 2X with 125 µl of 2.5mg/mL N^6-Methyladenosine-5'-monophosphate sodium salt (CHEM-IMPEX INT'L INC., Wood Dale, IL). Supernatant was added to Trizol-LS followed by RNA isolation as per manufacture's protocol. Final RNA sample was brought up in 10 µl of water.

PCR for MeRIP

Reverse transcription was performed on 10 µl m6A PolyA$^+$ RNA from the MeRIP with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). After diluting cDNA two-fold, quantitative real-time PCR was performed using a Roche Lightcycler 96 with Fast Start Essential DNA Green (Roche Diagnostics Corporation, Indianapolis, IN) and primers from Integrated DNA Technologies, Inc. (Coralville, Iowa). Primer efficiency

was verified to be over 95% for all primer sets used. Quantification of mRNA from the MeRIP was carried out via ΔCT analysis against non-immunoprecipitated input RNA. All real-time PCR primer sets were designed so the products would span at least one intron (>1kb when possible), and amplification of a single product was confirmed by agarose gel visualization and/or melting curve analysis.

siRNA Transfections

Either a negative siRNA (Silencer; Life Technologies, Carlsbad, CA) or HIF-1α and HIF-2α siRNAs (Silencer; Life Technologies,) transfected together using Lipofectamine RNAi Max 54 µl/plate as per manufacturer's protocol (Life Technologies) using 180 pM siRNA/10 cm dish. Cells were incubated for 72 hours post-transfection with the last 24 hours in either normoxic or hypoxic conditions.

Plasmid Transfections

Either a negative control plasmid shRNA scramble control, Alkbh5 psi-U6 shRNA construct (GeneCopoeia, Rockville, MD) or a Mettl3 and Mettl14 flag tagged construct given by Dr. Jing Crystal Zhao [13] was transiently transfected in immortalized and oncogenically transformed HMEC cells using Lipofectamine 2000 (Life Technologies). Cells were incubated for 48 hours post-transfection before scratch assays.

Scratch Assays

Cells were cultured in 6 well plates (USA Scientific) with MEBM supplemented media as described previously. Media was replaced with Serum free MEBM supplemented media

8 hours prior to the scratch. A scratch was made with a p200 pipette tip (USA Scientific) and media was removed and cell washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Corning) and fresh serum free MEBM supplemented media added. Wound healing was measured at 24 hours post-scratch.

Invasion Assays

100µl of a 1/3 matrigel membrane matrix (Corning) diluted in 0.01M Tris pH8, and 0.7% NaCl was layered in a 6.5 mm Transwell with 8.0µM Polycarbonate membrane insert (Corning) and incubated for 6 hours in 37ºC. $1x10^6$ Cells were resuspended in 200µl serum free media and plated on the membrane. 600µl of media with serum was added to the lower chamber cells were incubated for 24 hours. Invading cells were fixed with methanol and stained with 0.02% crystal violet.

siRNA's

siRNA	Product
siHIF-1α	Hs_HIF1A_5 S102664053, Qiagen FlexiTube
siHIF-2α	Hs_EPAS1_5 S102663038, Qiagen FlexiTube
Negative Control #1 siRNA	Ambion cat. # AM4635

Primer List

Gene	Forward (5'-3')	Reverse (5'-3')
Dusp1	CAACCACAAGGCAGACATCA	CAGTGGACAAACACCCTTCC
eEF1A1	CGGTCTCAGAACTGTTTGTTTC	AAACCAAAGTGGTCCACAAA
Glut1	TATCGTCAACACGGCCTTCACTGT	CACAAAGCCAAAGATGGCCACGAT
Jun	TTCTATGACGATGCCCTCAAC	TCAGGGTCATGCTCTGTTTC
VHL	TCTCTCAATGTTGACGGACAGCCT	GGTCTTTCTGCACATTTGGGTGGT

Antibodies

Antibody	Catalogue #	Vendor	WB Dilution	IP Amount
ZNF217	NBP2-46424	Novus Biologicals	1/1000	
YTHDF2	Sc-162427	Santa Cruz Biotechnology	1/200	
ALKBH5	HPA007196	Sigma Life Science	1/1000	
YTHDF1	116894	NovoPro	1/500	
METTL14	HPA038002	Sigma Life Science	1/1000	
METTL3/MT-A70	A301-567A	Bethyl Laboratories	1/5000	
YTHDC1	14392-1-AP	Proteintech	1/200	
FTO	SAB3500038	Sigma-Aldrich	1/1000	
N6-methyladenosine (m6a)	MABE1006	EMD Millipore		5 µg

Chapter 4

Discussion: The role of N^6-Methyladenosine in hypoxia and cellular transformation

The work outlined in this dissertation describes unique functions for the mRNA modification N^6-methyladenosine in hypoxia and breast cancer. Protein expression through translational regulation has been well studied in these and other similar systems. While understanding translational regulation has been useful, many protein expression phenomena in hypoxia and cancer have gone unexplained. Recently, research in these fields have started investigating post-transcriptional regulation in hypoxia and cancers, and in doing so have focused heavily on RBP and microRNAs that may alter the fate of the mRNA. The findings in this report indicate that an mRNA modification can also alter the fate of mRNAs and lead to changes in phenotype in a cell based model.

Post-Transcriptional regulation of mRNAs in hypoxia

Post-transcriptional regulation of mRNAs can be altered in response to changing cellular environments. Hypoxia is one such environment that causes global cellular changes including overall decreases in general translation efficiency. A hypoxic cellular environment leads to the discontinuation of transcription and translation of many mRNAs and proteins resulting in the reduction of protein expression. However, it is interesting that transcription and translation of some mRNAs continue. Because hypoxia is important for embryonic development, tumor formation, and other situations where low oxygen occurs, it is necessary to understand how this hypoxic regulation of protein expression occurs. As stated before, past research has discovered that HIF plays a large role in transcriptional regulation in hypoxia, and translational regulation in hypoxia is achieved through phosphorylation of eIF2α slowing the recruitment of ribosomes to mRNA and regulation of the cap binding protein eIF4F [139, 164, 165]. However, post-

transcriptional regulation of hypoxic mRNA has been understudied. While in normoxic conditions RNA-binding proteins and micro-RNAs have been identified as important factors in post-transcriptional regulation, determining the specific RBP's or micro-RNAs that may play a role in each specific cellular condition has been difficult. One explanation for these difficulties is the large number of RBPs and lack of knowledge of the consensus sequence or sequence specificity for these RBPs.

In this report, our focus has been on the mRNA modification m6A which only occurs within a specific consensus sequence. Only a limited number of RBPs can interact with this consensus sequence, and only a few RBP's binding potential are affected by the secondary structure changes caused by the m6A modification. In fact, a recent report determined many of the RBPs that are affected by the modification [166]. Based on this report, we can focus our attention on these specific RBPs to determine which one stabilizes mRNA through m6A in hypoxia as well as which RBPs have roles in other cellular conditions. Because of this, some of the previously discussed difficulties can now be addressed when studying the effects of the m6A mRNA modification on post-transcriptional regulation. While not all post-transcriptional regulation processes will be affected by RNA modifications, understanding the effects of mRNA modifications on mRNA may lead to a better path for studying post-transcriptional regulation in hypoxia and other cell stresses

Consensus sequence of m6A methylation

As stated earlier, m6A methylation in mRNA occurs at a DRACH consensus sequence primarily near both start and stop codons as well as a few sites in the coding sequence [23, 24, 56], but GGACU is the most common sequence found to be

methylated [167]. On average, each mRNA contains three actual m6A residues [61]. However, as one might imagine, not all consensus sequences are methylated at one given time. It seems possible, and likely, that the specific sites which are methylated may change in response to the cellular environment. It is possible that under hypoxic conditions, m6A sites switch from one consensus sequence to another. These potential switches in m6A sites could help explain the increase in m6A in hypoxic conditions. Perhaps m6A switches from a site located near a stop codon to a site in the middle of the coding sequence. This site switching of m6A might lead to switches in the RNA binding proteins that can bind the mRNA and may lead to differences in the fate of the mRNA. For example, this may cause m6A sites to be bound by YTHDF2 leading to degradation of the RNA, or it may switch sites to be bound by an RBP increasing mRNA stability. It would be possible to test the potential site switching through RNA sequencing methods.

For example, MeRIP sequencing protocols as well as other RNA sequencing protocols use sequencing reads of fragmented RNA which is mapped back to the fragment's parent RNA. This method would also allow us to determine which specific fragments of RNA are methylated in certain conditions. We could look at RNA from normoxic and hypoxic conditions to determine if there is a shift in the m6A landscape. For example, if we see m6A methylation in some targets mostly in the 3` end in normal conditions, but shift to the 5` end in hypoxic conditions we could conclude that hypoxia causes a shift in methylation sites. This type of experiment could allow us to determine the regulation of m6A methylation in hypoxia and other cellular conditions, and could lead to better explanations of m6A RBP competition. It seems possible that shifts in the

methylation sites would lead to different RBP binding allowing for different effects of m6A methylation.

m6A methylation dynamics

The results in this report show that m6A methylation levels change in response to a cellular stress condition. Based on these data we might conclude that m6A methylation is dynamically altered in response to hypoxia. However, recent reports have suggested that m6A may not be dynamic [21, 168]. In order to reconcile our findings with these reports, we must first define what each of us means by m6A dynamics. In the other report from the Darnell lab, they show that m6A levels do not change once pre-mRNA is methylated and enters the cytoplasm. They show that the sites that are methylated in the nucleus are still methylated and are the only methylated sites in the cytoplasm. This data suggests that both the demethylases and methyltransferase do not function in the cytoplasm.

This is not the same criteria that was used when we concluded that methylation is dynamically altered in response to hypoxia. We simply mean that m6A methylation is different in one condition compared to another. Still, it is possible that in hypoxic conditions, methylation does not change from its pre-mRNA stage to mature cytoplasmic mRNA. However, my results also do not rule out the possibility that this is occurring. In fact, the report suggesting that m6A methylation is not dynamic only measures m6A levels in one cell type and in one cellular condition. We might expect that cells continuing to grow in long-standing normal conditions might not have a need to dynamically alter their mRNA methylation. It seems likely that when comparing cell

types and other cell conditions, like hypoxia, methylation may change from nuclear to cytoplasmic mRNA.

In order to test this in hypoxia, we would need to run MeRIP sequencing on nuclear and cytoplasmic mRNA from both normoxic and hypoxic conditions. However, we could also test the possibility of m6A dynamics (by Darnell's definition) in any condition by determining if methylation or demethylation of mRNA can occur in the cytoplasm. I believe that these types of questions can be answered by future students in Dr. Mansfield's lab.

The effect of m6A methylation on RNA stability

A major focus of the Mansfield lab is the effect of hypoxia on post-transcriptional regulation. Originally, Dr. Mansfield and others found that hypoxia increased the stability of a subset of mRNAs [124], and set out to find an RNA binding protein that might facilitate the increases in RNA stability. Around this time, it was reported that the m6A modification caused a decrease in mRNA stability [6, 9]. For this reason, I decided to investigate m6A in hypoxia and determine if it played a role in the increased stability of mRNAs in hypoxia. Our initial hypothesis was that m6A levels would decrease in hypoxia leading to an increase in mRNA stability. However, as seen in this dissertation, hypoxia led to an increase in m6A methylation in HEK-293T cells (Figure 2.1) as well as immortalized and oncogenically transformed HMEC cells (Figure 4.1). Originally, this increase in m6A made little sense as m6A has been shown to decrease mRNA stability rather than increase mRNA stability as was seen in hypoxic conditions. However, we tested the effect of the m6A modification on mRNA stability in hypoxia and found that m6A actually increased mRNA stability in this system. It is interesting that many others

have shown the m6A modification leads to mRNA degradation whereas we have shown in hypoxia that m6A leads to mRNA stability. There are many possible reasons for these

Figure 4.1

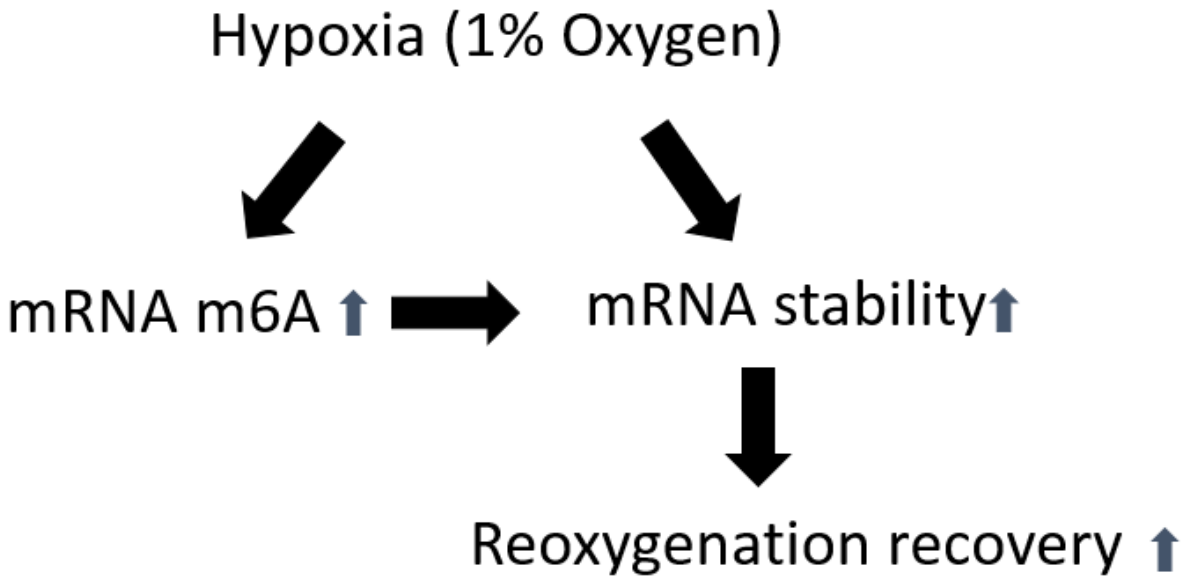


Figure 4.1 Model of m6A in hypoxia

One percent oxygen hypoxic stress leads to an increase in m6A levels in HEK-293T cells. This increase of m6A in hypoxia directly leads to stabilization of mRNA. Ultimately, this stabilization of mRNA led to quicker recovery after recovery from hypoxia.

discrepancies that are worth investigating in the future.

As stated previously, methylated RNA can be bound by a number of different RBPs, and these RBPs all have different functions. It is possible that in other systems, methylated mRNA is bound in majority by YTHDF2 which leads to degradation of mRNA, but in hypoxia a different RBP binds leading to mRNA stability. We attempted to observe any differences in m6A mRNA binding of RBPs including YTHDF2, YTHDF1, and YTHDC1. Unfortunately, we never were able to obtain any conclusive data. However, even if the m6A recognizing RBP were to change in hypoxic conditions, we must still ask ourselves why this might be occurring. Protein expression was measured and no differences in any of the m6A binding proteins were observed. It is also possible that the m6A binding proteins can be sequestered or activated in certain conditions. Additionally, it is possible that in hypoxic conditions, sites of m6A methylation were altered, as described in the previous paragraph, leading to differential outcomes in RBP competition.

The effect of m6A methylation on RNA stability in hypoxia may also have nothing to do with the currently identified m6A RBPs. m6A methylation has been shown to alter the secondary structure of mRNA [60]. It has also been found that this alteration of mRNA secondary structure has allowed additional RBPs to interact with the mRNA [58, 59]. It is possible that increased m6A methylation in hypoxia may lead to changes in the mRNA secondary structure which may attract additional RBPs that aid in mRNA stability, while repelling RBPs that lead to mRNA degradation.

m6A methylation of mRNA can decrease the stability of mRNA as shown by others, or increase stability of mRNA as we have seen in this report. mRNA methylation

is most likely able to regulate mRNA in both of these contradictory ways because of its effect on RNA secondary structure and recruitment of RBPs as described above. Overall, this would suggest that mRNA methylation is needed for fine tuning of protein expression in many cellular conditions.

m6A methylation in Breast Cancer

The ultimate goal of this project was to determine if a post-transcriptional regulation process had a phenotypic effect on breast cancer. We used a genetically defined breast cancer progression model developed by our collaborators in Jack Keene's lab at Duke University. In this model, m6A methylation decreases as cells progress to a cancerous phenotype, but m6A levels were increased back to primary cell like levels in hypoxia (Figure 4.2). These changes in m6A levels bring up numerous questions about the impact of m6A methylation on cellular phenotype. Firstly, why does m6A methylation decrease during breast cancer progression? Is the decrease in m6A because of the progression in the cancer phenotype, or does the decrease in m6A aid in the cancer phenotype? Based on our data in Chapter 3, it appears that the decrease in m6A methylation does not aid in the progression of the cancer phenotype as increasing m6A in the transformed cells further stimulates the cancer phenotype. However, due to technical issues we were never able to manipulate m6A in the primary cells through transient transfection methods. As these cells have shown they can be manipulated through a pCL retroviral vector system [163], it may be possible to manipulate m6A through this same system. We could then test if overexpressing or knocking down m6A in the primary cells led to a cancer like phenotype. Based on ours and other's data, I would hypothesize that manipulating m6A methylation would not lead

Figure 4.2

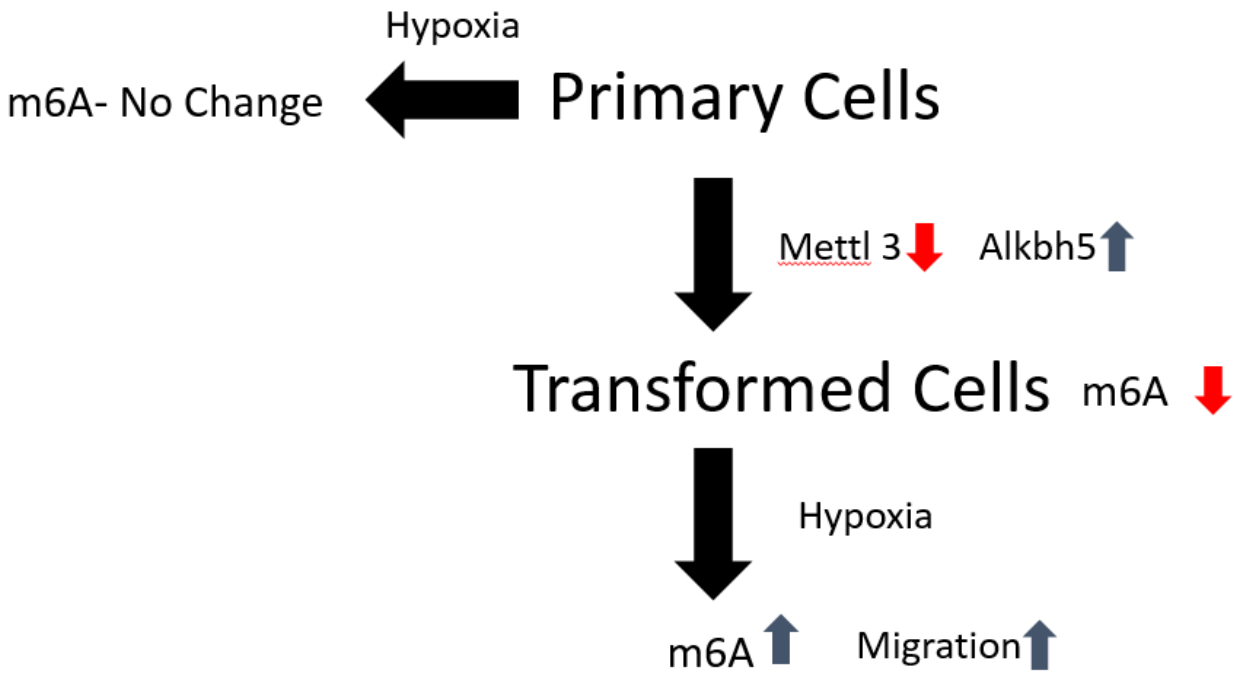

Hypoxia
m6A- No Change
Primary Cells
Mettl 3
Alkbh5
Transformed Cells
m6A
Hypoxia
m6A
Migration

Figure 4.2 m6A methylation in a breast cancer progression model

m6A levels decrease in immortalized and transformed HMEC cells. This decrease in m6A is due to a decrease in the methyltransferase Mettl3 and an increase in the demethylase ALKBH5. m6A levels in the immortalized and transformed lines then increase in hypoxic conditions. The increase in m6A in hypoxia leads to an increase in cell migration.

to a cancer like phenotype, as we and others have seen that m6A itself does not lead to cell progression, but rather seems to fine-tune the current process already in place.

The second question we must ask is why does m6A levels increase in hypoxic conditions. In fact, this increase in m6A directly contradicts previous reports that show m6A methylation decrease in breast cancer stem cells in hypoxic conditions [15]. There are many reasons that might suggest why our data is contradictory to theirs. First, their report measured m6A methylation in total RNA rather than mRNA. In fact, I would find it difficult to make any conclusions about m6A in mRNA from their report given that mRNA makes up a small percentage of total RNA. Additionally, they did not measure m6A methylation using LC-MS which has become the gold standard for measuring global m6A levels, but rather used an antibody-based colorimetric methylation quantification kit that is rather inexpensive and readily accessible but is still rarely used in the field due to poor results. However, they did notice a decrease of m6A in a specific mRNA, Nanog, using the MeRIP technique. Unfortunately, we were not able to confirm these results in any of our cell types as Nanog was not expressed in the cell types used in our project. Based on these facts, there are many possibilities as to why our data is contradictory with this previous report, but we still must answer how m6A is increasing in hypoxic conditions in our model system.

Based on our data (Figure 3.6), m6A methylation rates in newly transcribed RNAs were not increased in hypoxic conditions. Therefore, either m6A is dynamic in the cytoplasm (even though other reports suggest that m6A is not dynamic as I explained previously in this chapter) or there is simply a buildup of stabilized methylated mRNA as demonstrated in chapter 2 (Figure 2.9). In addition, the increase in m6A in hypoxia

appears to be regulated by HIF as knockdown of HIF decreased m6A methylation in a number of specific mRNA targets. Interestingly, in the previous report that was mentioned earlier has conversely shown that HIF knockdown increased m6A levels in Nanog mRNA. Once again, these results could not be confirmed in our model as Nanog is not expressed, but it seems likely that HIF is regulating m6A methylation, but different mRNA messages are regulated in opposite ways. Overall, it appears that according to our data, m6A methylation is increased in hypoxic conditions due to a HIF-mediated response in which m6A methylated mRNAs are stabilized.

Our data has also shown that m6A alone is able to phenotypically alter our transformed HMEC cells. Overexpressing the m6A methyltransferase or knocking down the demethylase ALKBH5 and thereby increasing m6A levels led to increased wound healing and invasion. Once again, the previous report showed that increased m6A levels through ALKBH5 knockdown impaired breast tumor formation. While we did not directly measure tumor formation, these two data sets again seem to be at odds. Keep in mind however, that our report, and this previous report are using two different models. While we are using a cancer progression model, this previous report used a breast cancer stem cell model. It seems likely that the m6A modification affects different stages of cells in opposing ways. Keep in mind, however, that m6A acted in contrasting ways in different stages of embryonic stem cells as described in chapter 1. In those reports, it appeared that m6A was simply fine-tuning the current cell stage rather than regulating progression of the stages. It is for this reason we need to investigate m6A methylation in all cell stages and not just end stage cancers.

Conclusions

The results from this dissertation project have aided the ever-growing m6A methylation and hypoxia fields in many ways. As stated before, previous reports had shown that m6A methylation led a decrease in mRNA stability. The work in this report proved that m6A methylation does not always lead to mRNA degradation, but rather may actually lead to increased mRNA stability depending on the situation. In addition, this report also revealed methods by which mRNA is stabilized under hypoxic conditions. It had been previously reported that hypoxia led to stabilization of a subset mRNAs, but these reports failed to conclusively find how these mRNAs were being stabilized. The data shown in this dissertation indicates that an increase in the m6A modification leads to stabilization of at least a handful of these specific mRNAs. This report also measured m6A methylation in a breast cancer cell progression model, and to my knowledge, is the first to show m6A levels decrease after progression from a normal primary cell to a cancer cell line in breast cancer or any other cancers. Excitingly, this project also determined that manipulation of the mRNA modification alone is enough to alter the phenotype of breast cancer through increased wound healing and invasion. Overall, my research has shown that m6A methylation does not have just one defined function in every cell condition, but rather fine-tunes protein expression and phenotypic changes through post-transcriptional regulation.

Future Directions

The importance of m6A methylation in many cellular processes is still not very well known. This field of study is still in its infancy, and I believe that there are many other avenues of focus for this project that will lead to many revealing results for the m6A field. Previously in this chapter I have described a few major questions in the field that deserve attention. I will summarize those potential directions for future projects as well as talk about a few others.

As stated above, the question of the m6A modification being dynamically regulated is very important to the m6A field right now, and I believe that hypoxia is the perfect model to answer this question. Using hypoxia, we may be able to determine if m6A levels can be altered in mature mRNA. In order to test this, it must first be established that methylation and demethylation can occur within the cytoplasm. Secondly, it must be determined if m6A methylation sites remain the same in nuclear in cytoplasmic cells when comparing cells grown in normal conditions to those in hypoxic conditions. In my opinion, it seems likely that m6A methylation is dynamic when cellular environments are changed.

Another important question that I think needs to be answered is which RBP is stabilizing m6A methylated mRNA in hypoxia. We know that YTHDF2 leads to degradation of m6A mRNA, but which RBP leads to stabilization? It is possible that an m6A binding protein or another RBP that binds due to the secondary structure rearrangements through the m6A modification may lead to mRNA stabilization. A recent paper has discovered a number of RBPs that the m6A modification attracts [166]. It seems very possible to determine which of these RBPs may result in hypoxic mRNA

stability through m6A. One would first have to determine which of these RBPs affects mRNA stability in hypoxia, and show a switch between its binding and a loss of YTHDF2 binding in hypoxia. I would propose that the hypoxic increase in m6A leads to secondary structure changes allowing for a non-m6A binding RBP to increase stability of the mRNA. It seems less likely that an m6A binding protein is able to have this affect as many of their functions are already known.

These previous two future directions projects are very mechanistic in nature. However, it is also important to continue investigating m6A methylation in breast cancer. I feel that it is necessary to repeat many of the findings in this report in other breast cancer types to fully understand m6A's role in breast cancer. Additionally, it is interesting that increased m6A in the immortalized HMEC cell line does not lead to the same phenotype as increased m6A in the oncogenically transformed cell line. One benefit of this system is that we know that the only difference between these two cells is the activation of hRAS in the oncogenically transformed line. It is therefore quite possible that the increase in m6A is working in conjunction with RAS or one of its downstream effectors. A major question that needs to be answered is how increased m6A after RAS activation leads to increased wound healing and invasion. It seems possible that increased m6A levels in specific RAS regulated mRNAs involved in migration may lead to greater expression of these proteins. Additionally, it is very important to determine the effects of m6A in breast cancers in an animal model. While the results in the breast cancer model in this report is a great starting point, we can only begin to fully appreciate the effect of m6A in cancer after animal model studies.

The projects I have outlined in this future directions section are, in my opinion, the most logical next steps in m6A research based on the results from my project. I believe the results I have obtained during these last 4 years in the Mansfield lab have influenced and impacted the RNA modification field and have provided a strong foundation for future investigations. It is my hope that the continuation of these lines of research will lead to valuable information that can someday be used to develop novel treatments for breast cancer and other diseases leading to better outcomes for human health.

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