REGULATION OF m^6A mRNA METHYLATION AND STEM CELL PLURIPOTENCY THROUGH GSK-3

by

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ABSTRACT

Glycogen Synthase Kinase 3 (Gsk-3) is a critical serine/threonine kinase known to regulate a diverse array of cellular functions including gene expression, cell differentiation, and cellular proliferation. Two different isoforms are present in mammalian cells, Gsk-3α and Gsk-3β, and they are almost exclusively negative regulators through phosphorylation of their target substrates. Two key pathways known to be affected by Gsk-3 activity are the insulin and Wnt signaling pathways. Both pathways are constitutively active in Gsk-3α−/−; Gsk-3β−/− embryonic stem cells (ESCs). These mouse ESCs deficient in Gsk-3 have been shown to remain in a pluripotent state capable of indefinite self-renewal, even in the absence of leukemia inhibitory factor (LIF). Gsk-3 inhibition has also been shown to promote the formation of induced pluripotent stem cells (iPSCs).

N^6^-methyladenosine (m^6^A) is the methylation of adenosine at the nitrogen-6 position, and is the most abundant internal mRNA modification. Although it was discovered more than forty years ago, very little is know about the function and regulation of this modification. An RNA methyltransferase, Mettl3, and an RNA demethylase, FTO, have been discovered recently, showing that the m^6^A modifications are both dynamic and reversible. It was also revealed that ESCs lacking Mettl3 remain in a highly pluripotent state and are unable to undergo differentiation, suggesting that m^6^A
also plays a key role in stem cell pluripotency. The phenotypic similarities between *Gsk-3α/Gsk-3β* double knockout (DKO) ESCs and *Mettl3−/−* ESCs, led us to hypothesize that Gsk-3 is playing a direct role in the regulation of m\(^6\)A. A direct connection between Gsk-3 and the m\(^6\)A modification on mRNA would have profound consequences for understanding how different Gsk-3-dependent signaling pathways regulate the balance between pluripotency and differentiation in embryonic stem cells.

The form and content of this abstract are approved. I recommend its publication.

Approved: Christopher Phiel
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<tbody>
<tr>
<td>3’ UTR</td>
<td>3’ Untranslated Region</td>
</tr>
<tr>
<td>AGi</td>
<td>Ascorbic Acid and a Gsk-3β inhibitor supplement</td>
</tr>
<tr>
<td>Alkbh5</td>
<td>AlkB Homolog 5</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>BMP2</td>
<td>Bone Morphogenetic Protein 2</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone Morphogenetic Protein 4</td>
</tr>
<tr>
<td>BPD</td>
<td>Bipolar Disorder</td>
</tr>
<tr>
<td>CKIα</td>
<td>Casein Kinase I-alpha</td>
</tr>
<tr>
<td>Erk1</td>
<td>Extracellular Regulated Kinase 1</td>
</tr>
<tr>
<td>Erk2</td>
<td>Extracellular Regulated Kinase 2</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>Esrrb</td>
<td>Estrogen-Related Receptor Beta</td>
</tr>
<tr>
<td>FGF4</td>
<td>Fibroblast Growth Factor 4</td>
</tr>
<tr>
<td>FTO</td>
<td>Fat Mass and Obesity Associated Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>Gsk-3</td>
<td>Glycogen Synthase Kinase</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome Wide Association Study</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DKO</td>
<td>Double Knockout</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human Embryonic Kidney Cells</td>
</tr>
<tr>
<td>hESC</td>
<td>Human Embryonic Stem Cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>HOTAIR</td>
<td>HOX Transcript Antisense RNA</td>
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<td>iPSC</td>
<td>Induced Pluripotent Stem Cells</td>
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<tr>
<td>Klf4</td>
<td>Krüppel-like factor 4</td>
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<tr>
<td>LCMS/MS</td>
<td>Liquid Chromatography Tandem Mass Spectrometry</td>
</tr>
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<td>Lithium</td>
</tr>
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<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<td>LNC RNA</td>
<td>Long non-coding RNA</td>
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<tr>
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<td>Knockout</td>
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<tr>
<td>m$^6$A</td>
<td>N$^6$-methyladenosine</td>
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<td>MEK</td>
<td>Mitogen Activated and Extracellular-Regulated Kinase Kinase</td>
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<td>mESC</td>
<td>Mouse Embryonic Stem Cells</td>
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<td>messenger RNA</td>
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<td>Neuroblastoma Cells</td>
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<td>Non-coding RNA</td>
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<td>Neural Stem Cells</td>
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<td>Opti-MEM</td>
<td>Minimum Essential Media</td>
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<tr>
<td>p110*</td>
<td>Myristoylated form of p110 subunit</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PI(3,4,5)P3</td>
<td>Phosphatidylinositol-3, 4, 5-triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RIP</td>
<td>RNA Immunoprecipitation</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>snoRNA</td>
<td>Small Nucleolar RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>Tcf3</td>
<td>T-cell factor 3</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal Deoxynucleotidyl Transferase</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
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<td>YTHDF2</td>
<td>YTH Domain Family 2</td>
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CHAPTER I

INVolVEMENT OF GSK-3 IN REGULATION OF m^6A DEMETHYLASE, FTO

Gsk-3 Overview

Glycogen Synthase Kinase 3 (Gsk-3) is a kinase that is a key component in numerous intracellular pathways, with over 100 known substrates (Beurel, Grieco, & Jope, 2015). It is a highly conserved proline-directed serine/threonine protein kinase (Hooper, Killick, & Lovestone, 2008) that has two different isoforms, Gsk-3α and Gsk-3β, shown to regulate diverse cellular functions (Wu & Pan, 2010) such as structure, gene expression, mobility, and apoptosis (Jope, Yuskaitis, & Beurel, 2007). Gsk-3α and Gsk-3β are encoded by two independent genes (Woodgett, 1990), yet have highly similar kinase domains (98% identity) (Doble, Patel, Wood, Kockeritz, & Woodgett, 2007). These isoforms exhibit functional redundancy in most pathways; each isoform is able to compensate for absence of the other (Bartman et al., 2014; Huang & He, 2008). Typically a negative regulator, Gsk-3 is rare among kinases in that it is constitutively active in its basal state, and is ubiquitously expressed. Both Gsk-3α and Gsk-3β have a preferred consensus phosphorylation motif of S/T-X-X-S/T (Fiol, Mahrenholz, Wang, Roeske, & Roach, 1987). Most Gsk-3 substrates require pre-phosphorylation of target substrates at a carboxy-terminal serine or threonine before Gsk-3 phosphorylates the target substrate (Hooper et al., 2008).

Gsk-3 is widely expressed and highly active in resting cells but is inhibited in response to cellular signals, such as hormones and growth factors (Doble et al., 2007). Gsk-3 itself is regulated by four key mechanisms: phosphorylation, subcellular localization, formation of protein complexes containing Gsk-3, and the phosphorylation
state of Gsk-3 target substrates (Jope et al., 2007). Inhibition or genetic deletion of Gsk-3 isoforms leads to changes in gene expression (Bartman et al., 2014), alterations in DNA methylation (Meredith et al., 2015), and changes in stem cell pluripotency (Sanchez-Ripoll et al., 2013).

The involvement of Gsk-3 in the Wnt and insulin signaling pathways has been well established (Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995; Peifer, Pai, & Casey, 1994; Yost et al., 1996). In Gsk-3α−/−; Gsk-3β−/− mouse embryonic stem cells (mESCs), both pathways are constitutively active and ligand independent (Doble et al., 2007). In the insulin signaling pathway, insulin binding its receptor activates phosphoinositol-3 kinase (PI3K), which in turn activates Akt (Ding, Chen, & McCormick, 2000). Akt then phosphorylates serine-21 and serine-9 in free cytoplasmic Gsk-3α and Gsk-3β (Cross et al., 1995; Klippel et al., 1996), respectively, and these phosphorylated amino-termini fold back and inhibit Gsk-3 kinase activity by acting as a pseudosubstrate (Frame, Cohen, & Biondi, 2001). This results in de-phosphorylation of downstream substrates and elicits an increase in glycogen and protein synthesis (Doble & Woodgett, 2003).

Gsk-3 also has a pivotal role in Wnt signaling, being a key component of the multi-protein β-catenin destruction complex (Clevers & Nusse, 2012). In the absence of Wnt, Gsk-3 is active and able to keep cytoplasmic β-catenin at low levels through phosphorylation. Once phosphorylated by the destruction complex, comprised of Axin, Gsk-3, CKIα, and APC, β-catenin is ubiquitinated and targeted for degradation by the proteasome (Aberle, Bauer, Stappert, Kispert, & Kemler, 1997). When Wnt binds its receptor, Frizzled, Gsk-3 is physically displaced from the destruction complex (Zeng et
β-catenin is able to accumulate and translocate to the nucleus, leading to association with LEF/TCF family of DNA binding proteins (Korinek et al., 1997) and the transcription of Wnt target genes (Jansson, Kim, & Cheng, 2015). The inhibition of Gsk-3 relieves the suppression of these target genes (Wu & Pan, 2010).

Gsk-3 dysregulation has been found to be associated with various disease states, such as obesity, inflammation, neurological disorders, neurodegenerative disorders and diabetes (Wu & Pan, 2010). Therefore, inhibiting or knocking out Gsk-3 will help provide insight into the precise role for Gsk-3 in these diverse diseases. Therefore, it is not surprising that several Gsk-3 small molecule inhibitors have been discovered, and are used to probe the biological function of Gsk-3, although none of these inhibitors can discriminate between the two isoforms (Coghlan et al., 2000). Examples of commonly used inhibitors include lithium chloride (LiCl), SB-415286 and CHIR99021. SB-415286 and CHIR99021 inhibit Gsk-3 through ATP competition (Bain et al., 2007; Coghlan et al., 2000; Murray et al., 2004), while LiCl functions by displacing magnesium, a required cofactor for Gsk-3 function (Klein & Melton, 1996). It is noted that LiCl has been a common treatment for bipolar disorder for over 60 years (Meijer, Flajolet, & Greengard, 2004), yet it remains to be determined whether the therapeutic effects of lithium are due to Gsk-3 inhibition or a Gsk-3-independent mechanism.

While small molecule inhibitors are widely used to probe the function of Gsk-3, no small molecule is specific, raising the possibility of off-target and dose-dependent effects, making the interpretation of data more difficult. For this reason, our lab has used a variety of methods that rely on either genetic deletion of Gsk-3 or tonic inhibition of Gsk-3 via the ectopic activation of specific signaling pathways. For example, stable
expression of the p110\(\alpha\) catalytic subunit of PI3K (termed p110\(^*\)) in wild type mouse ESCs leads to the phosphorylation and inhibition of Gsk-3 and constitutive activation of the insulin signaling pathway (Popkie et al., 2010). Gsk-3 double-knockout (\(Gsk-3\alpha^{-/-}; Gsk-3\beta^{-/-}\)) mouse embryonic stem cells (Doble et al., 2007) allow for more effective downstream functional studies without the potential for off-target inhibitor effects.

**Pluripotency in Mouse Embryonic Stem Cells**

Mouse embryonic stem (ES) cells are derived from the inner cell mass (ICM) of pre-implantation blastocysts (Evans & Kaufman, 1981) and are characteristically pluripotent; each cell has the capability to differentiate into all cell types derived from the three embryonic germ layers: ectoderm, mesoderm, and endoderm (Burdon, Smith, & Savatier, 2002; G. J. Pan, Chang, Scholer, & Pei, 2002). ESCs are capable of indefinite self-renewal in vitro under appropriate culturing conditions (G. Pan & Thomson, 2007). Given the ability of pluripotent stem cells to differentiate into virtually any cell type, intense research efforts have been focused on somatic cell reprogramming and its potential in the treatment of many devastating diseases (Salomonis et al., 2010). Despite these efforts, there remain holes in our knowledge about the precise mechanisms involved in maintaining stem cells in a pluripotent state (G. Pan & Thomson, 2007).

Development of the embryo is controlled by coordinated action of signaling pathways, extrinsic factors, and transcription factors (Sanchez-Ripoll et al., 2013). Somatic cell reprogramming has previously been accomplished by nuclear transfer into oocytes (Pomerantz & Blau, 2004) or by fusion with ES cells (Ishikawa et al., 2006). This indicated that embryonic cells contained factors that could confer pluripotency to
somatic cells (Patel & Yang, 2010). In a seminal series of experiments, Takahashi & Yamanaka demonstrated that overexpression of specific transcription factors (Oct4, Sox2, Klf4, c-Myc) are capable of reverting differentiated cells into stem cells, termed induced pluripotent stem cells (iPSCs) ((Nakagawa et al., 2008; Takahashi & Yamanaka, 2006). Transcription factors activate or repress expression of downstream genes when exposed to environmental and developmental signals (Kadonaga, 2004). These cells exhibit the characteristic cell surface markers, express the same genes, and have the same differentiation potential as ES cells (Takahashi & Yamanaka, 2006).

The so-called Yamanaka factors (Oct4, Sox2, Klf4, c-Myc), have been studied in-depth to better understand how they act as master regulators of pluripotency. Oct4, also known as Oct3, is a member of the POU (Pit-Oct-Unc) transcription factor family and is expressed almost exclusively in ES cells (Okamoto et al., 1990). Oct4 often binds in partnership with Sox2. Studies show that Oct4, Sox2, and Nanog, another transcription factor shown to promote pluripotency (Mitsui et al., 2003), bind to many of the same promoter sequences and target common core signaling pathways that are intricately connected in governing pluripotency (Loh et al., 2006). One key transcriptional target of Nanog is Esrrb (Estrogen-Related Receptor β) (Festuccia et al., 2012). Esrrb is required for suppressing differentiation and lies downstream of Gsk-3 and Tcf3 (Martello et al., 2012). These transcription factors and networks work synergistically in a highly regulated manner to control and maintain stem cell pluripotency.

Effect of LIF/STAT Pathway on Stem Cell Pluripotency

Under early, commonly used cell culturing conditions, ES cells tended to spontaneously differentiate rather than undergo continuous self-renewal. Leukemia
inhibitory factor (LIF) was discovered to be a key factor that could promote the maintenance of self-renewal of mouse ES cells in vitro (G. Pan & Thomson, 2007). LIF is a member of the interleukin-6 cytokine family and binds to a heterodimeric receptor, consisting of the LIF receptor and gp130. Binding of LIF to its receptor activates downstream signaling pathways through gp130, such as STAT3 (signal transducer and activator of transcription 3), PI3K, and Ras/Erk pathways (Cartwright et al., 2005).

Activation of the STAT3 pathway leads to dimerization and translocation of STAT3 into the nucleus, where it binds promoters and enhancers of target genes (Graf, Casanova, & Cinelli, 2011). When the STAT3 pathway is disrupted, ES cells undergo differentiation. Alternatively, artificial activation of the STAT3 pathway can maintain self-renewal of ES cells, even in the absence of LIF (Tai & Ying, 2013). The LIF/STAT3 signaling pathway is one of the only known pathways involved in mESC pluripotency (Smith, 2001). However, this pathway is expendable in ES cells before gastrulation, suggesting other signaling cascades are vital for ESC renewal (Sato et al., 2003).

Both the activation of the STAT3 and the PI3K pathway positively regulates self-renewal. Alternatively, the activation of the Ras/Erk pathway leads to ES cell differentiation into endoderm lineage. As LIF activates all three pathways, balance must be fine-tuned in order to achieve LIF-mediated maintenance of ESC self-renewal (Storm et al., 2007). LIF is often supplemented with Bone Morphogenetic Protein 2 or 4 (BMP2/4) in culture medium to better sustain pluripotency (Sanchez-Ripoll et al., 2013).

**Effect of Gsk-3 Inhibition on Stem Cell Pluripotency**

Inhibition of Gsk-3 has also been shown to enhance and sustain stem cell pluripotency (Sanchez-Ripoll et al., 2013). It was discovered that simultaneous inhibition
of Gsk-3 and mitogen-activated protein kinase kinase (MEK, also known as MAPKK), was sufficient to achieve ground state pluripotency. The combination of these two inhibitors was deemed the 2i cocktail (Ying et al., 2008). Furthermore, mESCs that are devoid of both Gsk-3 isoforms are resistant to differentiation (Doble et al., 2007; Sanchez-Ripoll et al., 2013). Two signaling pathways regulated by Gsk-3 activity, Wnt (Wray et al., 2011) and insulin (Sanchez-Ripoll et al., 2013), have both been implicated in the maintenance of stem cell pluripotency, yet the precise role for Gsk-3 in these pathways remains unclear.

**N⁶-methyladenosine mRNA modification**

There are over 100 known RNA nucleotide modifications, many of which play important regulatory roles, similar to that of dynamic DNA and protein modifications (He, 2010). N⁶-methyladenosine (m⁶A) is the methylation of the N⁶ position of adenosine and is a post-transcriptional mRNA modification (Grosjean, 2005). It is the most abundant and dynamic internal eukaryotic mRNA modification. m⁶A is roughly 0.1%-0.4% of total adenosine residues in mRNA (Meyer et al., 2012). Through m⁶A-seq, it was revealed that m⁶A modifications cluster around the stop codon, within long internal exons (Dominissini et al., 2012), and within the 3’ untranslated region (Meyer et al., 2012). m⁶A-seq was performed using a human hepatocellular carcinoma cell line and identified roughly 12,000 m⁶A sites within 7,000 coding genes and 250 non-coding; conversely, many transcripts were completely devoid of m⁶A (Dominissini et al., 2012). m⁶A is highly evolutionarily conserved between the mouse and human, with many of the same orthologous mRNAs methylated, suggesting important biological function for m⁶A modification (Dominissini et al., 2012).
The addition of m$^6$A is catalyzed by Mettl3 (methyl-transferase like 3) and Mettl14 (Lee, Kim, & Kim, 2014), which are components of a larger methyltransferase complex (J. Liu et al., 2014); Mettl3 is believed to be the primary catalytic component of this complex. The m$^6$A modification is reversible, and is regulated by RNA demethylases. Two m$^6$A demethylases have been identified, FTO and Alkbh5 (Jia et al., 2011; Zheng et al., 2013). FTO, the fat mass and obesity associated protein, was one of the first genes identified via a genome wide association study (GWAS) to contain a single nucleotide polymorphism (SNP) associated with obesity in humans (Loos & Yeo, 2014). SNPs in the first intron are associated with increased body mass index and increased risk for diabetes (Keller et al., 2011). The association is thought to be mediated by insulin signaling, but the true mechanism still remains largely unknown. It has also not been explained whether aberrant m$^6$A levels result from the presence of the SNP associated with obesity.

Four out of five YTH domain family proteins have been confirmed for the selective binding of the m$^6$A modification through a conserved hydrophobic pocket (Li, Zhao, Wu, & Shi, 2014). YTHDF2 was one of the first proteins found to recognize and bind m$^6$A (Wang et al., 2014). Binding of YTHDF2 has been shown to regulate RNA decay through localization of the methylated mRNAs to the P-bodies, where mRNA is then degraded (Wang et al., 2015). Knockdown of YTHDF2 results in roughly a thirty percent increase in mRNA half-life of methylated targets when compared to non-targets (Wang et al., 2014). This could serve to adjust the expression of regulatory genes. YTHDF1 directly promotes translation of m$^6$A mRNAs (Wang et al., 2015). m$^6$A has also been shown to alter the local structure of mRNA and long-noncoding RNA
(IncRNA). It has been shown to be essential for development and cell viability, yet its mechanisms are still poorly understood (N. Liu et al., 2015).

N^6-methyladenosine (m^6A) mRNA modification effect on stem cell pluripotency

Thousands of mRNAs show conserved m^6A modifications, including those encoding transcription factors required for maintaining pluripotency. mRNAs encoding Nanog, Klf4, and Myc all contain m^6A tags (Batista et al., 2014). Methylation of these transcription factor encoding mRNAs could lead to a reduced mRNA half-life. Mettl3 was recently identified as a regulator of termination of naïve pluripotency through a small interfering RNA screen (Geula et al., 2015). In pre-implantation embryos, pluripotent stem cells are referred to as 'naive', and they become 'primed' during post-implantation development, through activation of differentiation markers and repression of pluripotency genes (Weinberger, Ayyash, Novershtern, & Hanna, 2016). Genetic deletion of Mettl3 leads to severe decrease of m^6A (Batista et al., 2014). After being grown in differentiation media for twenty-one days, Mettl3^-/- mESCs failed to up-regulate developmental markers and repress pluripotency genes, such as Nanog and Esrrb (Geula et al., 2015). Furthermore, differentiation defects could be rescued through introduction of WT Mettl3 allele (Batista et al., 2014). This suggests that levels of m^6A dynamically effect stem cell pluripotency.

Specific Aims

The main objective of this research project is to study the relationship between Gsk-3 and stem cell pluripotency by a novel mechanism: the regulation of the N^6- methyladenosine (m^6A) modification on mRNA. We postulate that the m^6A modification is regulated by Gsk-3 through phosphorylation of the protein FTO. FTO is a known m^6A
demethylase and when phosphorylated, the protein would be marked for ubiquitination and degradation. This is mechanistically similar to the role of Gsk-3 in the regulation of β-catenin. Inhibition of Gsk-3 would result in accumulated FTO protein levels, leading to reduced m^6^A levels and increased stem cell pluripotency. Identifying the regulatory role of m^6^A and FTO through Gsk-3 may provide a strong mechanistic understanding of the involvement of Gsk-3 in stem cell differentiation.

**Involvement of Gsk-3 in m^6^A Regulation**

Aim #1: Quantify levels of m^6^A methylation in wild-type and Gsk-3 DKO mouse embryonic stem cells using liquid-chromatography, mass spectrometry.

Aim #2: Determine phosphorylation status of FTO through liquid-chromatography, mass spectrometry and phosphoenrichment.

**Materials and Methods**

**Observing Protein Expression:**

**Protein Isolation**

1. Cell media was removed with vacuum and cells were washed with 500ul 1x PBS.

2. Cells were lysed with 350ul IP lysis buffer with 1% NP-40 and Sigma Protease Inhibitor Cocktail at 1:1000 using rubber policeman.

3. Lysates were incubated on ice for 30 minutes, vortexing thoroughly every 10 minutes.

4. Lysates were centrifuged at 14,000 RPM for 15 minutes at 4°C in order to pellet cellular debris.

5. Supernatant was transferred to a fresh microcentrifuge tube, used immediately, or stored at -20°C.
**Western Blotting**

For FTO westerns, nitrocellulose membrane was blocked in 5% milk/TBST for 1 hour at room temperature while rocking. Primary FTO antibody (PhosphoSolutions Reference#: 597-FTO) was added to the membrane at 1:1000 in 10mL of 1% milk/TBST. Membrane was incubated in primary antibody overnight at 4°C while rocking. Membrane was then washed with TBST (1x for 15 min and 2x for 5 min) and Anti-Mouse secondary antibody (GE Healthcare Reference #: NA931V) was added at 1:10,000 in 10mL of 1% milk/TBST for 30 minutes at room temperature while rocking. Membrane was washed with TBST (3x for 5 minutes) and then ECL (Amersham ECL) was added to membrane for 5 minutes. Membrane was blotted dry and then imaged on ChemiDoc XRS+ (Biorad).

For α-tubulin westerns, membrane was blocked in 4% BSA/TBST for 1 hour at room temperature while rocking. Primary α-tubulin antibody (Cell Signaling Reference#: A11126) was added at 1:1000 in 10mL of 4% BSA/TBST. For secondary antibody, Anti-Mouse was added at 1:20,000 in 10mL of 4% BSA/TBST. Similar procedure was followed as used in FTO westerns.

For Gsk-3α/β westerns, membrane was blocked in 5% BSA/TBST for 1 hour at room temperature while rocking. Primary Gsk-3α/β antibody (Calbiochem Reference #: 368662) was added to membrane at 1:1000 in 10mL 5% BSA/TBST. For secondary antibody, Anti-Mouse was added at 1:20,000 in 10mL 5% BSA/TBST. Similar procedure was followed as used in FTO westerns.

For V5 westerns, membrane was blocked in 5% milk/TBST for 1 hour at room temperature while rocking. Primary V5-HRP conjugated antibody (Invitrogen Reference
#: R961-25) was added to membrane at 1:1000 in 10mL 5% milk/TBST. No secondary antibody needed. Similar imaging procedure was followed as used in FTO westerns.

For Flag westerns, membrane was blocked in 3% milk/TBST for 1 hour at room temperature while rocking. Primary Flag antibody (Sigma Reference #: F3165) was added to membrane at 1:1000 in 10mL 3% milk/TBST. For secondary antibody, Anti-Mouse was added at 1:20,000 in 10mL 3% milk/TBST. Similar procedure was followed as used in FTO westerns.

Over-Expression of FTO and Mettl3 in Mammalian Cells

Gibson Assembly Cloning of FTO V5 AND METTL3 FLAG into pCAGEN

1. Mammalian expression vector of interest, pCAGEN, was linearized using 2 uL of EcoR1 restriction enzyme, 2uL EcoR1 Buffer, 2 ug of circular pCAGEN, and 14 uL dH2O, to make the final reaction volume 20 uL. The digest reaction was incubated at 37°C for 15 minutes.

2. 5’ phosphate groups on pCAGEN were removed in order to prevent plasmid recircularization. 1 uL of antarctic phosphatase (NEB) was added to the digest reaction. Samples were incubated at 37°C for 15 minutes.

3. The effectiveness of the digest reaction was confirmed by running the digested product on a 1% agarose gel. The circular plasmid was added as a control.

4. After validation of successful digestion, the remaining linear plasmid was purified using the QIAquick Gel Extraction Kit from QIAGEN.
a. 1 volume of isopropanol was added to remaining digest reaction. Solution was added to spin column in a collection tube. Tube was spun at 10,000 rpm for 1 minute and the flow through was discarded.

b. The wash was performed by adding 750 uL of PE Buffer to the spin column. Tube was spun at 10,000 rpm for 1 minute and the flow through was discarded.

c. Empty column was re-spun at 10,000 rpm for 1 minute.

d. The spin column was placed in a fresh collection tube. 30 uL Buffer EB was added to the spin column and the tube was spun at 10,000 rpm for 1 minute to elute purified digest product.

5. The synthesized gBlock gene fragments (IDT, sequences contained in Appendix) were resuspended according to IDT protocol.

a. The tube was centrifuged for 5 sec at 3000 xg.

b. Sterile TE buffer (pH 7.5) was added to a concentration of 10ng/uL.

c. Solution was homogenized by vortexing and incubated at 50°C for 20 minutes.

d. Sample was placed on ice for immediate use. (Eventually stored at -20°C).

6. A 3-fold molar increase of DNA fragments containing 15 bp overlaps and 50 ng of linear plasmid was combined for the assembly (5-fold molar excess of fragment was used if the DNA was less than 200 bp). DNA fragments were converted into pmoles using Equation 1.
7. The Gibson assembly was prepared on ice by combining 50ng linear pCAGEN vector, 3-fold molar excess fragments, and 10 uL Gibson Assembly Master Mix. Milli-Q H2O was added to make a total volume of 20 uL.

8. Samples were incubated at 50°C for 15 minutes (samples that contained more than 3 fragments were incubated at 50°C for 30 minutes). After incubation, samples were immediately placed on ice or stored at -20°C.

9. NEB DH5-alpha competent E. coli cells (included in Gibson Assembly Kit) were transformed with 2 uL of assembly reaction. Overnight cultures were made from colonies on LB + ampicillin plates. Plasmids were purified via the GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to protocol.

10. To confirm if fragments were successfully cloned into pCAGEN, restriction digests were performed and products were run out on a 1% agarose gel.

**N2A and HEK293T Mammalian Cell Transfection Using PEI**

1. Day 1: Cells were plated at 1.0x10^6 cells per well in 6-well plates in cell specific media (Appendix).

2. Day 2: Media was not changed in 6-well plates. 2.0 ug of total DNA (1800 ng plasmid of interest + 200 ng pMax-GFP) was added to 100 uL Opti-MEM per transfection. No DNA was added to untransfected control well. In a separate tube, 5 uL of PEI was added to 95 uL Opti-MEM per transfection. Tube was tapped to mix and incubated at room temperature for 5 minutes. 100 uL PEI:Opti-MEM was transferred to DNA:Opti-MEM tubes. Tubes were tapped to mix, centrifuged at lowest speed for collection, and incubated at room
temperature for 20 minutes. Mixture was added to cells drop-wise. Cells were incubated at 37°C with 5% CO₂ overnight.

3. **Day 3:** To determine if transfection was successful, presence of green fluorescence from pMax-GFP was observed using fluorescence microscopy.

### mESC Transfection Using PEI

1. Mouse ESCs were washed, trypsinized, and resuspended in Opti-MEM. 10 uL of resuspension was added to 10 uL trypan blue dye. The total number of ESCs was counted using the Cell Countess (Invitrogen).

2. **1 x 10⁶** cells were aliquotted into 2 mL microcentrifuge tubes, one per well for a six-well plate. Tubes were centrifuged for 2 minutes at 1500rpm. Supernatant was carefully aspirated.

3. Cell pellets were resuspended in 300 uL Opti-MEM.

4. 2 ug of total DNA was added to the resuspended cells (200 ng pMax-GFP and 1800 ng plasmid of interest). No DNA was added to untransfected control cells. Cells were mixed by careful pipetting.

5. 100 uL of PEI was added to the resuspended cells. Cells were mixed by pipetting.

6. Cells were incubated for 30 minutes at room temperature. Mixture was pipetted every 10 minutes.

7. The transfection reaction was plated drop-wise on freshly prepared gelatin-coated 6-well plates with 2mL warm and complete ESC media (Appendix).

8. Transfection was incubated overnight at 37°C and visualized the next day using the fluorescence microscope to confirm GFP expression.
Quantification of m\(^6\)A Levels in Mammalian Cells

**RNA Isolation**

RNA was isolated using Direct-zol RNA Miniprep by Zymo. 500uL of TRIzol was added per well of a 6-well plate to begin lysing process (1mL of TRIzol was added per 10cm plate). RNA isolation followed kit protocol. All isolated RNA was quantified using Nanodrop 2000 (ThermoScientific).

**mRNA Enrichment**

1. 50 ul of oligo-d(T) magnetic beads were aliquotted to fresh microcentrifuge tubes, one per RNA sample.

2. 200 ul of Lysis/Binding Buffer (miRVANA RNA Isolation Kit) was added to each tube and samples were vortexed and mixed on rotator for 2 minutes at room temperature.

3. 5ug of isolated total RNA was added to magnetic beads. Tubes were placed on rotator overnight at 4°C.

4. Tubes were placed on magnet plate for one minute and supernatant was saved as “oligo-dT other” for further analysis.

5. 100 ul RNase-free H\(_2\)O was added to tubes, vortexed, and placed back on magnet, discarding H\(_2\)O. Wash was repeated two times.

6. In order to elute RNA from beads, 20 ul cold 10mM Tris-HCl at pH 7.5 was added to beads and the tube was incubated at 80°C for 2 minutes.

7. Tube was returned to magnet and RNA was immediately placed in new tube on ice.
8. RNA was quantified using the Quibit HS RNA Assay Kit (ThermoFisher Scientific) and stored in the -20°C.

**mRNA Digestion**

1µg of poly-A mRNA was digested by 4ul nuclease P1 (2 U) in 40 ul of LCMS/MS buffer (Appendix) at 37°C for 1 hour. After incubation, 3ul of ammonium bicarbonate (1 M) and 0.5 ul alkaline phosphatase (0.5 U) was added. An additional incubation at 37°C for 1 hour was performed. To confirm digestion, samples were run out on a 1% agarose gel with an undigested RNA sample as a control. Samples were transported to Anschutz Medical Campus for analysis using LCMS/MS. Levels of adenosine and methyl-adenosine were reported.

**Dot Blot**

Nitrocellulose membrane was rehydrated in H₂O for 3 minutes. The membrane was then placed in the Bio-Dot Microfiltration Apparatus (BioRad). Each well H₂O and flushed by gentle vacuum. 5ul of H₂O was added with 5ug of total RNA. RNA was allowed to bind membrane via gravity until all solution had flown through. The membrane was removed from the apparatus and cross-linked using UV light (Stratalinker). The membrane was placed back in the apparatus and blocked for 10 minutes using sterile TBST with 5% milk. Primary Anti-N6-methyladenosine antibody (Milipore Reference #: ABE572) was added to membrane at 1:500 in at room temperature for 1 hour in 5%milk/TBST. Membrane was then washed four times with TBST. For secondary antibody, Anti-Rabbit was added at 1:5,000 for 30 minutes at room temperature in 5% milk/TBST. The membrane was washed four times with TBST and imaged using Pierce ECL Western Blotting Substrate.
Quantification of Endogenous FTO and Mettl3 in Mammalian Cells

**RNA Isolation**

RNA isolated using mirVana mirRNA Isolation Kit by Life Technologies followed kit protocol. When RNA was to be isolated using Direct-zol RNA Miniprep by Zymo, 500uL TRIzol was added per well of a 6-well plate to begin lysing process (1mL of TRIzol was added per 10cm plate). RNA isolation followed kit protocol. All isolated RNA was quantified using Nanodrop 2000 (ThermoScientific).

**cDNA Synthesis**

cDNA was synthesized using High Capacity Reverse Transcriptase kit (Applied Biosystems) according to manufacturers protocol. Total RNA, 1ug- 2ug, was used to synthesize cDNA. The amount of input RNA used was kept constant for each set of RNA samples.

**qPCR**

To quantify gene expression, three biological replicates were created for each cell or treatment type. Total RNA was isolated from cells and cDNA was synthesized using 1ug-2ug RNA. All cDNA samples were diluted to 8.89ng/ul using DEPC H2O. A master mix for each probe was made by combining 2x TaqMan Mix (with UNG), 20x TaqMan probe, and DEPC H2O. Master mix components were multiplied by total number of wells designated for each probe. After 19.38uL of master mix was added to each designated well, 5.63 uL of diluted cDNA was added to plate in triplicates in order to have three technical replicates of each sample. Plates were spun down for 30 seconds at 2,000 RPM to ensure all components were combined at the bottom of the plate, and then placed in
StepOne qPCR Machine (Applied Biosystems). Data was collected by running the Comparative C\textsubscript{T} (\Delta\Delta C\textsubscript{T}) program, which followed the cycling parameters:

\begin{center}
\begin{tabular}{|c|c|}
\hline
qPCR Program: & \\
\hline
2 min & 50°C x 1 cycle \\
10 min & 95°C x 1 cycle \\
[15 sec & 95°C] \\
1 min & 60°C x30 cycles \\
\hline
\end{tabular}
\end{center}

Detection of FTO Phosphorylation in Mammalian Cells

**Overexpression of exogenous FTO-V5**

WT and Gsk-3 DKO mESCs were transfected with 2000 ng of FTO containing a V5 epitope tag, referred to as FTO-V5, according to protocol previously described. Total protein was isolated and successful transfection was confirmed through western blot using a primary antibody to the V5 epitope tag.

**V5 Immunoprecipitation**

Remaining protein lysate, 300 ul, was added to 2 ul of V5-agarose beads (Bethyl Laboratories Reference #: S190-119). Lysates were incubated with beads for 2 hours at room temperature while rotating. Lysates were centrifuged at 3000 x g for 60 seconds at 4°C, in order to pellet beads. Pellet was washed 4 times with IP lysis buffer and Sigma Protease Inhibitor Cocktail. Protein was eluted by adding 40 ul of 1x Tris-tricine sample buffer and incubated for 5 minutes at 100°C. Beads were pelleted by centrifugation at 14,000 rpm for one minute. Supernatant was transferred to a fresh micro-centrifuge tube and stored at -20°C until use. Successful immunoprecipitation was confirmed through western blot using an antibody to the V5 epitope tag.

**Protein staining and LCMS/MS**
V5 immunoprecipitated protein samples were run out on 7.5% polyacrylamide Mini-PROTEAN TGX Precast Gel (BioRad). The polyacrylamide gel was then stained with Imperial Protein Stain (ThermoScientific). FTO-V5 stained protein band was identified and excised. Samples were transported to Anschutz Medical Campus for analysis using LCMS/MS.

**Phosphoprotein enrichment**

Total protein was isolated from WT and Gsk-3 DKO mESCs that were transfected with FTO-V5. Samples were enriched for phosphoproteins using the Pro-Q Diamond Phosphoprotein Enrichment Kit (Invitrogen Reference #: P33358) according to manufacturer’s protocol for nondenatured protein lysates.

**Results**

Several studies have shown that inhibition of Gsk-3 increases stem cell pluripotency, even in the absence of LIF, but the exact mechanisms and pathways involved remain to be determined. The same differentiation resistant phenotype is seen in mESCs lacking the RNA methyltransferase, Mettl3, leading us to hypothesize that there may be a mechanistic connection between Gsk-3 inhibition and regulation of RNA methylation that dictates pluripotency. It was shown in Mettl3 KO ESCs that reduced levels of m$^6$A mRNA methylation leads to an increase in the half-life of those mRNAs, which notably includes many pluripotency-related genes. These same pluripotency genes are also increased in Gsk-3 DKO ESCs. Therefore, we hypothesized that Gsk-3 is regulating the m$^6$A mRNA modification. Because Gsk-3 is almost exclusively a negative regulator, we proposed Gsk-3 is regulating m$^6$A through controlling the levels of FTO protein via phosphorylation, much as it regulates β-catenin in the Wnt pathway.
Phosphorylation of FTO would subsequently lead to ubiquitin-mediated degradation in WT ESCs. Therefore, when Gsk-3 is inhibited or genetically deleted, FTO would accumulate, and m^6A levels would decrease. This decrease of m^6A would result in the increased half-life of specific mRNAs, leading directly to enhanced pluripotency.

Over-Expression of FTO and Mettl3 in Mammalian Cells

In order to determine the precise mechanism by which Gsk-3 affects m^6A, we wanted to overexpress both the m^6A demethylase, FTO, and the m^6A methyltransferase, Mettl3, into mouse embryonic stem cells. We first needed to clone these protein coding sequences into a mammalian expression vector. Gibson Assembly cloning (Gibson, 2011) was used to insert each of these sequences into mammalian expression vector, pCAGEN. gBlock gene fragments (IDT) are double-stranded, sequence verified nucleotide fragments that were synthesized to be identical to FTO and Mettl3 protein and contained amino-terminal V5 and Flag epitope tags, respectively (sequences in Appendix). An epitope tag was used to facilitate the detection of each protein by using epitope specific antibodies, as well as to distinguish between endogenous and exogenous protein. Gibson Assembly requires that each fragment contain 15-20 bp overlaps that correspond with the expression vector. Subsequently, 15 bp overlaps complementary to pCAGEN linearized with EcoRI were added to each end of the synthesized DNA fragments. Gibson assembly mastermix contains three key enzymes, an exonuclease, DNA ligase, and DNA polymerase to create a sealed double-stranded plasmid, with the gBlock inserted in proper orientation. OneShot chemically competent cells were transformed with Gibson Assembly product and plasmid DNA was isolated through mini-preps (GeneJet). A restriction digest was performed to screen clones in order to ensure proper insertion of the
gBlock. Clones with proper restriction enzyme digest bands (Figure 1A and 1B) were subjected to Sanger sequencing provided by Eton Bioscience to further confirm correct cloning.

After successful cloning of Mettl3 and FTO, we wanted to confirm that these constructs would express in mammalian cells. Mouse embryonic stem cells were transfected with 1800ng of plasmid DNA and 200 ng of GFP using polyethylenimine (PEI) as a transfection agent (Bartman, Egelston, Ren, Das, & Phiel, 2015). Mouse embryonic stem cells were chosen in order to study the effect of manipulating m^6A levels on pluripotency. Therefore, mESCs were an ideal model. Successful transfection was visualized through presence of GFP.

In order to verify successful overexpression of these plasmids in mESCs, protein was isolated and subjected to western blot against an untransfected control protein lysate. Antibodies to V5 and Flag epitope tags were used (Figure 2A and 2B). Both constructs produced robust protein expression, indicating we were able to overexpress these proteins in mouse embryonic cells.
Figure 1. Verification of Successful Gibson Assembly Cloning. (A) BglII restriction digest of pCAGEN-FTO-V5 to ensure that transcripts were inserted correctly into mammalian expression vector pCAGEN. 900bp fragments were expected in correct clones. (B) EcoRV restriction digest of pCAGEN-Mettl3-Flag to ensure proper cloning. 800bp fragments were expected in correct clones.
Levels of Endogenous FTO in Mammalian Cells

Our hypothesized mechanism proposes that Gsk-3 regulates FTO through phosphorylation, resulting in ubiquintation and degradation of FTO. Therefore, we wanted to quantify levels of endogenous FTO in order to determine if FTO protein levels were indeed higher in Gsk-3 DKO mESCs due to the inability of FTO to be phosphorylated by Gsk-3 and subsequently marked for degradation. Under normal ESC culturing conditions, with the addition of LIF, no difference was seen between levels of protein in WT compared to Gsk-3 DKO mESCs (Figure 3). These results were expected because the addition of LIF maintains WT ESCs in a pluripotent state. Gsk-3 DKO ESCs
are able to maintain high pluripotency levels regardless of LIF addition. Therefore, we concluded that optimized cell culturing conditions was necessary in order to separate pluripotency mechanisms from the effects of LIF addition.

A previous study indicated that Gsk-3 DKO ESCs were able to retain pluripotency even after removing LIF for 14 days (Doble et al., 2007). We decided to implement a similar regimen, culturing WT and Gsk-3 DKO mESCs in complete ESC media (Appendix) in the absence of LIF for fourteen days. Cells were washed and treated with new media daily. On day 14, protein was isolated and quantified. We then performed a western blot to assess levels of FTO and α-tubulin (Figure 4). A robust decrease of FTO was seen in WT ESCs when compared to Gsk-3 DKO ESCs. α-tubulin served as a loading control to indicate relative levels of protein. This data strongly supports our hypothesis that Gsk-3 DKO ESCs have accumulated levels of FTO protein through lack of FTO degradation.

In order to rule out possible effects on FTO protein due to changes in gene expression, we examined FTO gene expression in these same cells. In addition to protein, total RNA was also isolated (Zymo) and cDNA was synthesized (Applied Biosystems). FTO gene expression was quantified through qPCR (Figure 5). Results indicate that relative gene expression remain constant, with a minor decrease in the Gsk-3 DKO cells. This is consistent with our hypothesis that regulation of FTO occurs post-transcriptionally.
Figure 3. FTO Protein Expression in WT and Gsk-3 DKO mESCs Cultured in Complete ESC Media in the Presence of LIF. Protein band sizes are indicated on left side. α-tubulin western served as a loading control.

Figure 4. FTO Expression in mESCs Cultured in Complete ESC Media in the Absence of LIF for 14 Days. FTO protein expression was observed in WT and Gsk-3 DKO mESCs that were grown in the absence of LIF for 14 days. The α-tubulin western was used as a protein loading control.
Effect of Gsk-3 Inhibition on FTO Protein Expression

Because we determined that FTO protein levels were significantly reduced in WT mESCs when compared to Gsk-3 DKO mESCs, we wanted to determine if treating WT ESCs with a Gsk-3 inhibitor would also be sufficient to prevent degradation of FTO protein in the absence of LIF. In order to determine the effects of Gsk-3 inhibition on FTO levels, WT cells were grown without LIF for fourteen days as previously described. WT cells were treated with various concentrations of known Gsk-3 small molecule inhibitors, LiCl and SB-415286 on day 14 and 15. On day 16, protein was isolated from

Figure 5. FTO Gene Expression in mESCs Cultured in Complete ESC Media in the Absence of LIF for 14 Days. FTO gene expression was observed in WT and Gsk-3 DKO mESCs that were grown in the absence of LIF for 14 days using qPCR.
WT cells and FTO and α-tubulin western blots were performed to access levels of protein present.

Results indicated that FTO protein levels increased with increased concentrations of SB415286, with the most robust expression seen in cells treated with 15µM SB415286 (Figure 6A). The LiCl treated cells showed a similar trend in increasing FTO expression levels with increasing LiCl concentrations, with the most robust expression seen in cells treated with 10mM LiCl (Figure 6B). These data show that Gsk-3 inhibition can mimic the effects on FTO protein expression that we observed in Gsk-3 DKO ESCs.
Quantification of m\textsuperscript{6}A levels in mESCs

Our hypothesized mechanism suggests that Gsk-3 regulates FTO through phosphorylation. It has already been well established that FTO is the m\textsuperscript{6}A demethylase. If Gsk-3 is knocked-out and FTO protein accumulates, the overall levels of m\textsuperscript{6}A should be reduced. Therefore, we wanted to quantify levels of m\textsuperscript{6}A from Gsk-3 DKO and WT cells. WT and Gsk-3 DKO cells were grown without LIF for 14 days. On day 14, total RNA
was isolated from both cell types and was enriched for mRNA using oligo d(T) magnetic beads. Each mRNA sample was then quantified via Quibit and 500 ng of mRNA was digested into single nucleotides using nuclease P1. Samples were sent to the Anschutz Proteomics Core and nucleotide bases were quantified via LC-MS/MS. Results indicated that there is approximately a 50% decrease of N\textsuperscript{6}-methyladenosine in Gsk-3 DKO mESCs. This strongly supports our hypothesis that Gsk-3 activity is regulating the levels of m\textsuperscript{6}A in mESCs.

Figure 7. Quantification of m\textsuperscript{6}A in WT and Gsk-3 DKO cells. WT and Gsk-3 DKO mouse embryonic cells were cultured in complete ESC media in the absence of LIF for 14 days. On day 14, total RNA was isolated. mRNA was enriched and digested into single nucleotides. Samples were analyzed using LCMS/MS in order to quantify both N6-methyladenosine and unmethylated adenosine.
Effect of Mettl3 and FTO Overexpression on Pluripotency Genes in mESCs

We next wanted to quantify the expression of pluripotency genes when we overexpressed either FTO or Mettl3 in mESCs. Because decreased m\textsuperscript{6}A is associated with increased pluripotency and increased mRNA stability, we expected to see an increase in gene expression when we overexpressed FTO and a decrease in gene expression when we overexpressed Mettl3. We chose to observe Esrrb (estrogen receptor β) mRNA because it is known to be affected by m\textsuperscript{6}A methylation. We grew WT and Gsk-3 DKO mESCs in the presence of complete ESC media with LIF. We then transfected FTO-V5 into WT mESCs and Mettl3-Flag into Gsk-3 DKO mESCs. Total RNA was isolated 48 hours after transfection and cDNA was synthesized. Quantitative PCR analysis shows that Esrrb mRNA levels stayed relatively constant after either treatment. We do see a minimal increase in Esrrb in WT with FTO (Figure 8A) and a minimal decrease in Gsk-3 DKO with Mettl3 (Figure 8B), although neither is significant. We uncovered that 48 hour overexpression is not enough time to create a large impact on gene expression. We determined that a stable cell overexpressing Mettl3 or FTO would be a more sufficient way to determine gene expression levels.
Characterization of Axin\textsuperscript{-/-} and Axin\textsuperscript{+/+} mESCs

Because Gsk-3 is a key component in numerous intracellular pathways, we wanted to investigate the effect of those pathways on m\textsuperscript{6}A levels and FTO regulation. Gsk-3 is inhibited through activation of the Wnt and Insulin signaling pathways and both

Figure 8. Esrrb Gene Expression in mESCs with Overexpression of Mettl3 or FTO. (A) Esrrb expression was observed in WT and WT + FTO mESCs grown in complete ESC media with LIF addition. (B) Esrrb expression was observed in Gsk-3 DKO and Gsk-3 DKO + Mettl3 mESCs grown in complete ESC media with LIF addition.
pathways have also been implicated in stem cell pluripotency. We wanted to determine if activation of either the Wnt or Insulin pathways had an effect on levels of m\(^6\)A in mESCs. We first wanted to look at Wnt signaling using Axin\(^{-/-}\) and Axin\(^{+/-}\) mESCs that we received from Frank Costantini, whose lab originally cloned and characterized Axin, but had not previously been characterized. Deletion of Axin would result in a constitutively active, ligand independent Wnt signaling. In order to determine if Axin was knocked out, we examined levels of β-catenin protein. Axin is a key component of the β-catenin destruction complex in the Wnt pathway, along with Gsk-3, so β-catenin protein levels should be elevated in Axin\(^{-/-}\), similar to that of Gsk-3 DKO cells. Results from western blotting experiments indicated that β-catenin protein levels are not elevated in the Axin\(^{-/-}\) and Axin\(^{+/-}\) mESCs (Figure 9A). Expression appears to be lower than that of WT mESCs. This indicates that the β-catenin destruction complex is still intact and β-catenin is being rapidly degraded.

Next we wanted to quantify gene expression levels of a gene known to be affected directly by the Wnt pathway. Activation of the Wnt pathway elevates expression of Axin2, so we would anticipate higher levels of Axin2 in Axin\(^{-/-}\) and Axin\(^{+/-}\) mESCs. RNA was isolated from WT, Axin\(^{-/-}\), and Axin\(^{+/-}\) and cDNA was synthesized. QPCR was performed and Axin2 gene expression was quantified in the Axin\(^{-/-}\) and Axin\(^{+/-}\), using WT as a control. Increases in Axin2 are moderate and are not indicative of Axin being genetically deleted (Figure 9B). These results indicate that Axin is still functioning properly and cells were not used for further experimentation. Taken together, we speculate that since the Axin\(^{-/-}\) ESCs were derived more than 20 years ago, tubes may have been mislabeled and we were sent the wrong clones.
Figure 9. Characterization of Axin\(^{-/-}\) and Axin\(^{+/+}\) mESCs. (A) \(\beta\)-catenin protein expression was observed in WT, Axin\(^{-/-}\), and Axin\(^{+/+}\), and Gsk-3 DKO mESCs. \(\alpha\)-tubulin western was used as a protein loading control. (B) AXIN 2 expression levels were quantified in WT, Axin\(^{-/-}\), and Axin\(^{+/+}\) mESCs.
**Effect of PI3K Pathway on m\(^6\)A levels**

Because Gsk-3 plays an integral part in the insulin/PI3K pathway, we wanted to determine if activation of the pathway would result in an effect on m\(^6\)A levels. Therefore, we wanted to quantify levels of m\(^6\)A from p110* mESCs and WT mESCs. While Gsk-3 is fully functional in p110* cells, the p110 subunit is myristoylated, leading to constitutive activation of the insulin pathway and inhibition of Gsk-3. WT and p110* cells were grown without LIF for 14 days. On day 14, total RNA was isolated from both cell types and was enriched for mRNA using oligo d(T) magnetic beads. Each mRNA sample was then quantified via Quibit and 500 ng of mRNA was digested into single nucleotides using nuclease P1. Samples were sent to the Anschutz Proteomics Core and nucleotide bases were quantified via LC-MS/MS. Results indicated that there is no difference in levels of N\(^6\)-methyladenosine in p110* cells compared to WT (Figure 10). This suggests that there is no effect of insulin signaling on m\(^6\)A, therefore regulation does not likely occur through this pathway.
Phosphorylation of FTO

Up to this point, we have presented important evidence suggesting that Gsk-3 activity is important for regulating FTO protein levels as well as m^6^A levels. However, most of the evidence of a role for Gsk-3 in regulating FTO has been circumstantial. Therefore we sought to obtain more definitive evidence that FTO is phosphorylated by Gsk-3. We noted that mouse FTO contains a Gsk-3 consensus motif, \textit{SCEGSEDESEDES} \textsuperscript{257}, within its amino acid sequence. In addition, mass-spectrometry data revealed phosphorylation on these serine residues in mouse cells (http://www.cellsignal.com/common/content/content.jsp?id=phosphositeplus). Hence, we
focused on that consensus sequence as the putative Gsk-3 phosphorylation site. Since there is no phospho-specific antibody for these sites on FTO, we were unable to perform a simple western blot. Instead, we approached this question by utilizing a kit that selectively binds phosphorylated proteins to a proprietary resin, allowing for an enrichment of all phosphorylated proteins. We could then use antibodies to FTO (endogenous) or epitope tag (overexpressed) to evaluate the phosphorylation status of FTO in WT and Gsk-3 DKO ESCs.

A Phospho-Protein enrichment kit (Molecular Probes) was used to enrich for phosphorylated proteins from ESCs. We isolated total protein from WT and Gsk-3 DKO mESCs where FTO protein levels are similar (Figure 3) and subjected each lysate to phospho-enrichment (Figure 11). We also saved the flow-through for further analysis of unphosphorylated proteins. Both the enriched and flow-through samples were quantified and analyzed by western blotting (Figure 12A). Endogenous FTO is observed in both WT and Gsk-3 DKO phospho-enriched samples. We expected protein in WT due to Gsk-3 phosphorylation, but protein may be present in Gsk-3 because FTO is being ‘primed’ and therefore is being phosphorylated by another kinase. We also saw FTO protein in both samples of the flow-through, although Gsk-3 DKO cells has much more robust expression of protein in the flow-through. Because the levels of endogenous FTO are low, we decided to overexpress FTO-V5 and then subject the samples to phospho-enrichment. We transfected WT and Gsk-3 DKO mESCs with FTO-V5 and isolated total protein, then performed V5 immunoprecipitation on a WT and Gsk-3 DKO sample to confirm expression. Next we performed phospho-enrichment on each sample, with untransfected WT cells used as a control. Lysates were then subjected to western blotting
with a V5-specific antibody (Figure 12B). For phospho-enriched samples, robust FTO was seen in WT cells, but no protein was detected in Gsk-3 DKO samples. This indicates that FTO is phosphorylated in WT mESCs, but not in Gsk-3 DKO mESCs.

We next sought to determine the precise sites of phosphorylation on FTO. Our approach was to overexpress a tagged version of FTO in WT and Gsk-3 DKO ESCs, then immunoprecipitate FTO, and perform mass spectrometry to identify phosphorylated residues. Therefore, we overexpressed FTO-V5 through PEI transfection into both WT and Gsk-3 DKO mESCs, and then immunoprecipitated FTO-V5 using V5 magnetic beads. Western blotting was performed to ensure that the FTO-V5 was being expressed and that the immunoprecipitation was accomplished successfully (Figure 13). We were then able to run protein lysates through SDS-PAGE, stain the gel using Imperial Protein Stain, and excise the band from the gel. Samples were transported to Anschutz Proteomics Core for analysis via LC/MS. Results indicated that 730 proteins were observed in the samples, therefore nonspecific binding occurred during immunoprecipitation. FTO was indeed one of the observed proteins, but we only obtained 42% coverage of the complete protein sequence in WT cells (Figure 14A), while coverage in Gsk-3 DKO ESCs was a meager 14% (Figure 14B). No phosphorylation was detected in either sample. Although we have yet to determine the precise site(s) of phosphorylation on FTO, based on LC/MS data, we can narrow down possible sites to those that were not covered, 58% of the protein. In addition, we did not obtain any coverage of the Gsk-3 consensus motif in either sample, meaning that the data does not rule out the possibility that FTO is phosphorylated by Gsk-3 on this consensus motif. Further studies will be necessary to fully test this hypothesis.
**Figure 11. Phospho-Protein Enrichment Schematic.** Phospho-enrichment enriches for all phosphorylated proteins, while unphosphorylated proteins are discarded in the flow-through.
Figure 12. Phospho-Protein Enrichment. (A) Levels of endogenous FTO were analyzed from WT and Gsk-3 DKO mESCs from phospho-enrichment and phosphorylated depleted flow-through using an antibody specific to FTO. (B) Levels of overexpressed FTO-V5 were analyzed from WT and Gsk-3 DKO mESCs from phospho-enrichment and V5 immunoprecipitation. Untransfected WT was used as a negative control.

Figure 13. FTO-V5 Protein Overexpression in mESCs. FTO-V5 protein expression was observed through a V5 specific antibody in WT and Gsk-3 DKO mESCs.
Figure 14. FTO-V5 Protein LC MS/MS. LC/MS FTO coverage seen in (A) WT mESCs and (B) Gsk-3 DKO mESCs. Yellow highlight indicated amino acid sequence coverage.
Conclusions

Our data is consistent with our hypothesis that Gsk-3 contributes to the regulation of stem cell pluripotency through the regulation of FTO and subsequently, m^6^A mRNA modification. Due to highly similar phenotypic characteristics of Gsk-3 DKO and Mettl3 KO ESCs, we hypothesized that low levels of m^6^A contributes to increased stem cell pluripotency. We postulated that Gsk-3 negatively regulates the m^6^A modification through phosphorylation of FTO, the m^6^A demethylase, resulting in ubiquitination and degradation. FTO phosphorylation would result in low levels of cytoplasmic FTO, and subsequently increased levels of m^6^A, causing a more rapid degradation of m^6^A modified mRNAs in wild-type cells. Pluripotency genes with m^6^A modifications would be degraded through selective binding of YTHDF2, poising ESCs for differentiation. In order to maintain pluripotency in wild-type stem cells, serum and LIF must be used in culturing conditions. Alternatively, when is Gsk-3 is absent, phosphorylation is reduced and FTO levels are elevated, resulting in reduced m^6^A levels and increased pluripotency.

When initially looking at wild-type and Gsk-3 DKO mESCs, FTO protein levels were relatively constant. It was determined that culturing WT ESCs in the presence of LIF artificially enhanced pluripotency, therefore FTO levels remained high. Cell culturing conditions needed to be optimized in order to observe accurate FTO levels. ESCs cultured in complete ESC media in the absence of LIF for fourteen days allowed for optimal observation of FTO protein expression (developed by Jennifer Egelston, Phiel Lab). When WT and Gsk-3 DKO mESCs were grown under these optimized conditions,
FTO expression was greatly reduced in WT ESCs when compared to Gsk-3 DKO ESCs. This data supports our initial hypothesis that in WT ESCs, FTO protein levels are indeed regulated by Gsk-3. In addition, we looked at gene expression levels of FTO in WT and Gsk-3 DKO cells grown under optimized conditions. Levels of FTO mRNA were relatively constant, with a slight decrease seen in Gsk-3 DKO mESCs. This supports our hypothesis that the mechanism behind regulation of FTO occurs post-translationally, rather than at the transcriptional level. Furthermore, we were able to rescue FTO protein expression in WT mESCs with addition of a Gsk-3 inhibitor, supporting our hypothesis that Gsk-3 is contributing to the degradation of FTO. The addition of 10mM LiCl or 15uM SB for two days was optimal for rescue of FTO.

In order to confirm that Gsk-3 DKO cells had increased pluripotency, we compared gene expression levels of transcription factors implicated in maintaining pluripotency. We observed increased expression of Nanog, Essrb, and Oct4 in Gsk-3 DKO ESCs compared to WT ESCs, confirming that Gsk-3 DKO cells have increased pluripotency even in the absence of LIF for fourteen days (data not shown, J. Egelston, Phiel Lab).

We next wanted to quantify m$^6$A in both WT and Gsk-3 DKO cells. Since FTO protein levels are increased in Gsk-3 DKO ESCs, we expected to observe changes in levels of m$^6$A modifications on mRNA. Total RNA was isolated from WT and Gsk-3 DKO mESCs cultured in complete ESC media in the absence of LIF for 14 days. mRNA was enriched from total RNA using magnetic oligo dT beads. Collected mRNA was digested into single nucleotides using nuclease P1 in order to perform Liquid Chromatography Tandem Mass Spectrometry (LC MS/MS). LC MS/MS data showed
roughly a 50% reduction in Gsk-3 DKO ESCs compared to WT ESCs, substantiating our hypothesis that inhibition of Gsk-3 results in reduced \(m^6A\) mRNA methylation levels through the regulation of FTO.

To confirm that FTO is indeed being phosphorylated, we wanted to subject samples to phospho-enrichment utilizing a kit that selectively binds phosphorylated proteins to a proprietary resin. We subjected WT and Gsk-3 DKO mESCs to phospho-enrichment in order to look at endogenous FTO protein. FTO protein is observed in both WT and Gsk-3 DKO phospho-enriched samples. We believe this is due to FTO being ‘primed’ by another kinase before Gsk-3 is able to phosphorylate its target. We saw a much more robust protein expression in Gsk-3 DKO flow-through, than in WT ESCs, indicating that much of the FTO protein in Gsk-3 DKO cells is unphosphorylated. We also overexpressed FTO-V5 construct in WT and Gsk-3 DKO cells and subject those samples to phospho-enrichment. For phospho-enriched samples, robust FTO was seen in WT cells, but no protein was detected in Gsk-3 DKO samples. This indicates that FTO is phosphorylated in WT mESCs, but not in Gsk-3 DKO mESCs.

Next we wanted to determine the precise residues within FTO where phosphorylation occurs. We again overexpressed FTO V5, immunoprecipitated the protein, and subjected the samples to Liquid Chromatography Tandem Mass Spectrometry (LC MS/MS). Unfortunately, only 42% of coverage of FTO was achieved in WT ESCs and only 14% in Gsk-3 DKO ESCs. Phosphorylation was not detected in WT or Gsk-3 DKO ESCs but neither sample included coverage of the Gsk-3 consensus motif. We can narrow down our phosphorylation target site to that of the 58% of
coverage not determined. This still leaves the Gsk-3 consensus site as possible target. Further studies will be required.

Final Words

Gsk-3 is tremendously important kinase involved in regulation of numerous intracellular pathways. Uncovering another layer of Gsk-3 complexity through understanding of the mechanism behind m⁶A regulation could have large impacts on current knowledge of devastating diseases. When dysregulation of Gsk-3 or m⁶A occurs, similar disease phenotypes are produced. FTO variants have now been associated with Alzheimer’s disease, independent of FTO effect on diabetes. Knockdown of FTO decreases tau phosphorylation, a protein associated with fibrillar lesions in neurodegenerative disorders (Pitman RT, 2013). Remarkably, over-activity of Gsk-3 has been shown to lead to increased β-amyloid production and inflammatory responses, hallmark characteristics of AD (Hooper et al., 2008). Gsk-3 and FTO could be working through the same pathway, ultimately affecting Alzheimer’s disease through mRNA methylation.

Another key disease is type-II diabetes. Overexpression of Gsk-3 increases phosphorylation and down-regulation of insulin receptor substrate-1. Inhibition of Gsk-3 helps overcome the resistance to insulin and promotes the conversion of glucose to glycogen (Eldar-Finkelman & Krebs, 1997). FTO was identified in a human GWAS study linking FTO with higher body mass index and type-II diabetes. A direct link between diabetes and FTO has yet to be determined (Loos & Yeo, 2014).

The missing link could be Gsk-3’s regulation of m⁶A. Similar stem cell pluripotency characteristics, disease phenotypes, and supporting data have led us to the
conclusion that there is a strong association between FTO and Gsk-3. In-depth understanding of this mechanism could lead to potential downstream therapeutics. Our main focus as we move forward is determining the specific phosphorylation site(s) in FTO and to determine if the mechanism behind m$^6$A is similar in humans to that observed in mouse ESCs.
CHAPTER III
FUTURE DIRECTIONS

Our lab has established a connection between Gsk-3 and m6A. Further characterization is still required to validate our mechanism. Our first focus is determining the specific site at which FTO is phosphorylated. We have provided evidence that indicates FTO is phosphorylated in WT mESCs and phosphorylation is lost in Gsk-3 DKO mESCs, but we have yet to provide conclusive evidence that Gsk-3 is responsible for the phosphorylation. This may be determined by attempting LC MS/MS in hopes to achieve better coverage of the FTO protein. This would show that the phosphorylation is present at the Gsk-3 consensus site in WT and absent in the Gsk-3 DKO cells.

Future directions will also include further characterization of m6A mRNAs via methyl-seq. We will use oligo d(T) beads and m6A specific antibodies to enrich for mRNAs that contain the m6A tag in WT, p110*, and Gsk-3 DKO mESCs. We will use methyl-seq in order to identify specific mRNAs that are affected by deletion of Gsk-3 and the myristoylation of the p110 subunit. This will help us to determine if known regulators of stem cell pluripotency are indeed affected by loss of Gsk-3. A better understanding of methylated genes being up-regulated or down-regulated in Gsk-3 DKO mESCs may also give us a better understanding of each components role in various disease states.

Because m6A is associated with decreased mRNA stability through selective binding of YTHDF2, our lab will perform mRNA half-life studies by treating WT and Gsk-3 DKO cells with alpha-amanitin, an inhibitor of RNA polymerase II, after removing LIF from cell media for fourteen days. In Gsk-3 DKO mESCs, where m6A is greatly reduced, mRNA half-life should be greatly increased. We will do qPCR in order to
evaluate levels of mRNA expression of genes known to regulate stem cell pluripotency and genes that were identified using methyl-seq. Studies have already shown that Mettl3 KO cells have roughly 30% decrease in half-life in these methylated pluripotency genes. We hope to see the same decrease in Gsk-3 DKO mESCs.

We want to precisely determine which Gsk-3 pathway is affecting levels of m$^6$A. We will initially look at the Wnt and PI3K pathways because both pathways have also been implicated in stem cell pluripotency. Our lab will develop an Axin, a key component in the Wnt pathway, knockout cell line utilizing CRISPR guide RNAs specific to Axin. Characterization of Axin KO cells that we previously provided showed no increase in β-catenin protein expression and no increase in Axin2 gene expression. We concluded that Axin was still fully functional in these cells. We will then use Axin -/- and p110* cells, previously described, to investigate how inhibition of Gsk-3 through activation of upstream signaling events affects m$^6$A levels in mRNA. We will work to quantify levels of m$^6$A methylation in Axin KO cells. We did not see a change in m6A levels in p110*, but this pathway may still have some correlation with the regulation of m6A. Further investigation is required.

Lastly, we will look at FTO and m$^6$A levels in different human cell types. Gsk-3 is implicated in a plethora of human diseases. Insight into levels of m$^6$A in various human cell types could help determine if these disease states are altered by Gsk-3 inhibition and levels of m$^6$A. We would also want to determine if the mechanism is the same in human cells. Human FTO protein lacks the same Gsk-3 consensus site, although much of the rest of the protein is conserved (87%). A single serine in the consensus site is mutated to a
proline. The site still provides a viable Gsk-3 target but further experiments will be imperative.
REFERENCES


APPENDIX

Equations
Equation 1:
\[
\text{pmoles} = \frac{\text{(mass of DNA in ng) x (1000 pg/ng)}}{\text{(# of sense strand bp) x (650 Daltons)}}
\]

Buffers and Reagents
LC/MS Buffer consists of 25mM of NaCl and 2.5mM ZnCl₂.

Cellular Mediums
N2A media consists of 50% DMEM (10% BGS, 1% Penicillin Streptomycin, 1% L-Glutamine), 50% Opti-MEM, 0.5% Pen-strep, and 0.5% L-Glutamine.

HEK293T media consists of DMEM, 10% BGS, 1% Penicillin Streptomycin, and 1% L-Glutamine.

ESC Media consists of DMEM, 15% FBS, 1% Pen-Strep, 1% L-Glutamine, 1% Sodium Pyruvate, 1% Non-essential Amino Acids. 2-mercaptoethanol (β-me) and LIF were added fresh each time (1uL of 55mM β-me per 1mL of media and 1uL ESGRO LIF per 1mL media).

IDT GBlock Sequences

FTO V5:
ATCATTTTGCGAAGAGATGGGTAAGCCTATCCCTAACCCTCTCTCTCGTGCTCTGA
TTCTACGAGCGCGTCCAGACCGAAGCAGAGAAGCAGAAGGCTTCAAGAA
ACTGAGGCTCTTTGAGGAGCTTGAAGACACTTGGCTTCTCTTACCTGACCCTCA
AAGATGATGATGCTATCACGAGTGGCAAGCTGAAATACCCTAAACTGGTTTT
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CTGCACCTACAAGTACTTGAACACCAGAAGCAGACTCTCTCAGGTGCTTGCCCGGTG
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TTAAGACGAGAGCCTACAAAGCAGACTTTGCTTCTAAGACTATCTGGATCTCTCA
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ACAGCTATAGCTGCGAAGGCCTTCTGAGGATGAAATGAGGACGAGCTTGGGAGGCG
CGAAGGCAGATCCTGATACCTTGGCAGCGTCTTGGTTTTTAAAGATCTCTTGGGACA
TCGAGACA CACCAGGATTA ACAATCCCT CTTCACCAGGAG ACTGCTATTT CATG
CTGGATGAC CTTCAATAGCC ACCACAGCAGA CTGGTTTTGG GCTGGCACA
TCGGTTTATC CCTACCAC GTGAGTGGC AGGATGCT ACACAGAGC AATTGA
ATATCTTA GAAC GCTGTTG TGGCCGTA AACAGGATG

METTL3 Flag:
ATCATTTGGCA AAGATGAC TCAACAGGATGACGACAGAT AAGATTACAA
GATGACGATA AAGTGGCC ACAGTGGGACT ATCTCTCTA AAGTGCAGCG
ATGAGTGGCT TGGAAAGG AAGAGATG GACGACGAT

TCGAGACA CACCAGGATTA ACAATCCCT CTTCACCAGGAG ACTGCTATTT CATG
CTGGATGAC CTTCAATAGCC ACCACAGCAGA CTGGTTTTGG GCTGGCACA
TCGGTTTATC CCTACCAC GTGAGTGGC AGGATGCT ACACAGAGC AATTGA
ATATCTTA GAAC GCTGTTG TGGCCGTA AACAGGATG

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GCAATGATTGAGACTGTCCTCCTGTCGCCCAAGAACATTGGAATTATTGAGGCG
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**FTO V5 S259_271A:**
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