THE EFFECT OF SETD3 ON BETA-CATENIN AND CANONICAL WNT SIGNALING PATHWAY ACTIVITY IN MOUSE EMBRYONIC STEM CELLS

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ABSTRACT

THE EFFECT OF SETD3 ON BETA-CATENIN AND CANONICAL WNT SIGNALING PATHWAY ACTIVITY IN MOUSE EMBRYONIC STEM CELLS

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Mouse embryonic stem cells (mESCs) are self-renewing, pluripotent cells that can differentiate into endoderm, mesoderm and neuroectoderm. As differentiation requires deactivation of pluripotency factors and activation of germ layer specific genes, many epigenetic factors play an essential role. A previously performed shRNA screen showed that SETD3 which is a SET-domain containing epigenetic factor, is an essential factor for mesendoderm differentiation of mESCs. Our preliminary data demonstrated that when mESCs were grown in standard serum-containing mESC medium prior to directed differentiation, wild type mESCs can successfully differentiate into mesendoderm, while SETD3 knockout (setd3Δ) mESCs cannot. According to our preliminary data, we aimed to identify the effect of SETD3 on the levels and localization of β-catenin as well as on the canonical Wnt signaling pathway activity in mESCs. Proximity ligation assay revealed that SETD3 and β-catenin are interacting in mESCs, further supporting the role of canonical Wnt signaling pathway. Even though immunocytochemistry assay performed wild type and setd3Δ mESCs grown in the same medium did not show significant localization differences, western blot experiments performed with nuclear fractions showed that setd3Δ mESCs grown in standard serum-containing mESC medium had lower levels

of β-catenin than wild type mESCs. Additionally, luciferase assay results demonstrated that, regardless of the growth medium, absence of SETD3 results with significantly lower β-catenin transcriptional activity. Observed lower nuclear β-catenin levels and lower canonical Wnt signaling pathway activity in the absence of SETD3 might explain the mesendoderm differentiation defect of setd3Δ mESCs grown in standard serum-containing mESC medium.

Keywords: SETD3, Mouse Embryonic Stem Cells, β-catenin, Canonical Wnt Signaling Pathway

ÖZ

SETD3'ÜN FARE EMRİYONİK KÖK HÜCRELERİNDE BETA-CATENİN VE KANONİKAL WNT YOLAK AKTİVİTESİ ÜZERİNDEKİ ETKİSİ

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Fare embriyonik kök hücreleri (EKH) kendini yenileyebilen ve endoderm, mezoderm ve nöroektoderme farklılaşabilen, pluripotent hücrelerdir. Farklılaşma, pluripotensi faktörlerinin deaktivasyonu ve ilkel tabakaya özgü genlerin aktivasyonu ile gerçekleştirildiğinden epigenetik faktörler bu süreçte önemli bir rol oynar. Daha önce gerçekleştirilen shRNA taraması SET bölgesi içeren bir epigenetik faktör olan SETD3'ün mezendoderm farklılaşmasında önemli bir faktör olduğunu göstermiştir. Ön verilerimize göre fare embriyonik kök hücreler (EKH) mezo/endoderme farklılaştırılmadan önce standart serum içeren EKH besiyerinde büyütüldüğünde normal EKH'ler başarılı bir şekilde farklılaşırken SETD3 geni silinmiş (setd3Δ) EKH'ler aynı şekilde farklılaşamamaktadır. Ön verilerimize dayanarak bu projede SETD3'ün EKH'lerdeki β-catenin miktar ve lokalizasyonu ve kanonikal Wnt sinyal yolağı aktivitesi üzerindeki etkisini belirlemeyi amaçladık. Yakınlık ligasyon deneyi sonucunda kanonikal Wnt sinyal yolağının rolünü destekleyecek şekilde, SETD3 ve β-catenin'in EKH'lerde etkileşim içerisinde olduğunu gördük. Aynı besiyerinde büyütülen normal ve setd3Δ EKH'ler kullanılarak gerçekleştirilen immünositokimya deneyinde belirgin bir lokalizasyon farklılığı görülmese de çekirdek fraksiyonları kullanılarak gerçekleştirilen western blot deneyi sonucunda standart serum içeren EKH besiyerinde büyütülen setd3Δ EKH'lerin β-catenin seviyesinin, normal

EKH’lere göre daha düşük olduğunu gözlemledik. Ek olarak lusiferaz deneyi sonuçları büyütme ortamından bağımsız olarak setd3Δ EKH’lerin normal EKH’lere kıyasla önemli ölçüde daha düşük β-catenin transkripsiyonel aktivitesi gösterdiğini ortaya çıkarttı. SETD3 yokluğunda gözlemlenen çekirdekteki düşük β-catenin seviyesi ve düşük kanonikal Wnt sinyal yolağı aktivitesi standart serum içeren EKH besiyerinde yetiştirilen setd3Δ hücrelerin mezendoderme farklılaşma kusurunu açıklayabilir.

Anahtar Kelimeler: SETD3, Fare Embriyonik Kök Hücreleri, β-catenin, Kanonikal Wnt Sinyal Yolağı

To my loving, supporting family and friends

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LIST OF ABBREVIATIONS

ABBREVIATIONS	
AP axis	Anteroposterior Axis
APC	Adenomosis Polyposis Coli
ARM repeats	Armadillo Repeats
ATP	Adenosine Triphosphate
BCL9	B-Cell CLL/Lymphoma 9 Protein
BRD2	Bromodomain Containing 2
BSA	Bovine Serum Albumin
CBP/CREBBP	CREB Binding Protein
CK1α	Casein Kinase 1α
CoIP	Co-immunoprecipitation
CSNK1D	Casein Kinase 1δ
CTD	C-Terminal Domain
DEGs	Differentially Expressed Genes
Dkk1	Dickkopf Wnt Signaling Pathway Inhibitor 1
Dvl	Dishevelled
E-cadherin	Epithelial Cadherin
EMT	Epithelial-to-Mesenchymal Transition
EpiSC	Epiblast Stem Cells
FBS	Fetal Bovine Serum

FBXW7β	F-Box and WD Repeat Domain Containing 7β
FoxM1	Forkhead Box Protein M1
Fzd	Frizzled
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GRG/TLE	Groucho/Transducin-Like Enhancer of Split
GSEA	Gene Set Enrichment Assay
GSK3β	Glycogen Synthase Kinase 3β
H3	Histone 3
H4	Histone 4
H3K4me2	Histone 3 Lysine 4 Dimethylation
H3K36me2	Histone 3 Lysine 36 Dimethylation
HDAC1	Histone deacetylase 1
ICC	Immunocytochemistry
ICM	Inner Cell Mass
IP-MS	Streptavidin-based Immunoprecipitation and Mass Spectrometry
KMT	Lysine Methyltransferase
LEF	Lymphoid Enhancer Factor
LIF	Leukemia Inhibitory Factor
LRP	Low Density Lipoprotein Receptor-Related Protein
MEF	Mouse Embryonic Fibroblast
mESC	Mouse Embryonic Stem Cell

MitoMEF	Mitomycin C treated Mouse Embryonic Fibroblast
MEK	Mitogen-Activated Protein Kinase Kinase
NLS	Nuclear Localization Signal
NTD	N-Terminal Domain
Oct4	Octamer-Binding Transcription Factor 4
PCP	Planar Cell Polarity
PLA	Proximity Ligation Assay
PYGO2	Pygopus Family PHD Finger 2
RT	Room Temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
Rubisco-LSMT	Rubisco Large Subunit Methyltransferase
SAM	S-adenosyl-L-methionine
SET	Suppressor of variegation 3-9, Enhancer of zeste and Trithorax
TC	Tissue Culture
TCF	T-Cell Factor
TE	Trophectoderm
TF	Transcription Factor
TLE	Transducin-like Enhancer of Split
VEGF	Vascular Endothelial Growth Factor
WT	Wild type

CHAPTER 1

INTRODUCTION

1.1 Early Mouse Embryonic Development and Embryonic Stem Cells (ESCs)

Mice models are frequently used in research as they enable accurate modeling of biological events in humans. Similar to other mammals, a mouse zygote is formed as a result of fertilization of an ovum with a sperm (National Research Council and Institute of Medicine, 2002). After fertilization, zygote starts to perform mitotic divisions called cleavage to increase the cell number without an increase in the total cytoplasmic volume, forming ball-like structures called blastomeres (Aiken et al., 2004; Wamaitha & Niakan, 2018). At the 8-cell stage, after three rounds of cleavage, blastomeres become flattened and more compact as a result of newly formed tight, adherens and gap junctions (Chuva de Sousa Lopes & Mummery, 2009; Takaoka & Hamada, 2012; Wamaitha & Niakan, 2018; H. Wang et al., 2008; White et al., 2016). Polarized compact cells are called morula and it cavitates to form the blastocyst (Chuva de Sousa Lopes & Mummery, 2009; Huang et al., 2015). Even though first cell fate decision was believed to be made based on the positioning of the polarized cells at the 16-cell stage, recent research revealed that cell fate decision precedes the actual polarization event, as molecular differences were detected in blastomeres of four cell stage such as differential methylation of 17th and 26th arginine residues of histone 3 (H3R17me and H3R26me) or differential kinetics of Oct4. (Plachta et al., 2011; Takaoka & Hamada, 2012; Torres-Padilla et al., 2007). Blastocyst is composed of; trophectoderm (TE), the polarized outer region cells, inner cell mass (ICM), the cluster of nonpolar cells under TE, and the fluid filled region called blastocoel (Huang et al., 2015; National Research Council and Institute of Medicine, 2002; H. Wang et al., 2008). Mouse embryonic stem cells (mESCs) are derived from

the ICM of the blastocyst. They are pluripotent cells which have self-renewal ability (Huang et al., 2015; Wamaitha & Niakan, 2018). Pluripotency indicates that upon receiving specific extracellular signals, they are able to differentiate into one of the three primary germ layers which are endoderm, mesoderm, and neuroectoderm (Lu & Zhang, 2015; Paranjpe & Veenstra, 2015; Terzi Cizmecioglu et al., 2020; Q. L. Ying et al., 2003). Self-renewal ability enables them to proliferate indefinitely without differentiating into a primary germ layer, preserving their pluripotent state (Lu & Zhang, 2015). ESCs can be indefinitely cultured *in vitro* to model various biological events such as cell fate decision or developmental mechanisms.

1.2 Culturing ESCs in a Pluripotent State

To properly model embryonic development *in vitro,* mESC culture medium must enable preservation of the stem cell character as well as the pluripotency state. For this purpose, two different specialized techniques with different growth media are used. In the first technique, ESCs are grown in standard serum-containing mESC medium (15% serum) which is supplemented by leukemia inhibitory factor (LIF), on mouse embryonic fibroblast cells (MEFs) whose growth is arrested with either radiation or Mitomycin C treatment (mitoMEFs). Cytokines and growth factors that are essential for cell growth come from the medium. Several other inhibitors that prevent differentiation of cells and that are important for maintaining pluripotency are provided by the MEFs beneath the ESCs.

In the alternative technique, ESCs are grown in low serum-containing (4% serum) or serum-free medium which is supplemented with MEK1/2 inhibitor (PD0325901) and GSK3 inhibitor (CHIR99021) (2i medium) in addition to LIF. The advantage of this technique over the first is that it allows to maintain ESCs pluripotent in a serum-free medium and without the need for a mitoMEF layer. Therefore, there is no need to eliminate components that come from the serum-containing medium and the MEFs before performing further experiments (Q. Ying et al., 2008).

1.3 Epigenetic Regulations

In eukaryotes, DNA is found in a compacted state inside the nucleus, wrapped around both histone and non-histone proteins (J. Wang, Qiu, et al., 2018). Compaction is achieved in several levels, one of which is the formation of nucleosome structures. Nucleosomes are like "beads on a string" and each containing ~147 base pairs of DNA that is wrapped around a histone octamer as a left-handed helix (Baldi et al., 2020; Campos & Reinberg, 2009). Histones are highly conserved, small, basic proteins (Eirín-López et al., 2009). As they are basic, they can easily bind to DNA that has an acidic nature. A histone octamer consists of core histones, two sets of Histone 3 (H3) – Histone 4 (H4) dimers that are surrounded by two sets of Histone 2A (H2A) – Histone 2B (H2B) dimers. Another histone protein, Histone 1 (H1), is called a linker histone and it binds to the linker DNA that is found between two nucleosomes to enable further compaction as well as to mediate changes in the DNA methylation status and histone modifications (Handy et al., 2012; Y. Zhang et al., 2012).

Spacing of nucleosomes determine the state of chromatin which can be either euchromatic or heterochromatic. Euchromatic regions are loosely packed regions. As they provide enough space for transcription factor binding, genes in these regions are referred as actively transcribed genes (Godini et al., 2018). Heterochromatic regions on the other hand, contain densely packed chromatin. Due to tight packaging of DNA, there is either none or very low gene expression from these regions (Fedorova & Zink, 2008; Handy et al., 2012). Besides gene expression regulation, these regions also play an important role in preserving the genomic integrity since they are found in telomeric ends and centromeres (Fedorova & Zink, 2008; J. Wang, Eisenstatt, et al., 2018). As gene expression levels differ throughout developmental processes, euchromatic and heterochromatic regions dynamically change, and these changes are regulated by epigenetic factors that introduce a variety of modifications.

Epigenetic regulations are essential for the normal embryonic development as well as differentiation of various cell types in adult organisms. They are heritable alterations that cause changes in the gene expression patterns. Epigenetic regulations do not depend on a change in the DNA sequence, instead, they rely on DNA methylation, histone modifications, action of ATP-dependent nucleosome remodeling complexes and noncoding RNAs such as microRNAs (Biswas & Rao, 2018; Handy et al., 2012; L. Zhang et al., 2020).

DNA methylation is covalent attachment of methyl groups to C5 position of cysteine residues. S-adenosyl-L-methionine (SAM) is used as a methyl group donor and its addition onto DNA is mediated by DNA methyltransferases (DNMTs), while sequential activity of ten-eleven translocation (TET) enzymes are required for its removal. Presence of DNA methylation causes repression of gene expression (Handy et al., 2012; He et al., 2012; Okano et al., 1999; Spruijt & Vermeulen, 2014). However, it can also cause activation as DNA methylation can cause recruitment of transcriptional activators (Spruijt & Vermeulen, 2014).

N-terminus tails of core histones protrude from the core. Serine, lysine, and arginine residues found in these tails can be post-translationally modified to regulate the chromatin status. These various modifications affect the nucleosomes which in turn change the accessibility of DNA, the structure of chromatin, therefore, the gene expression from that region (Jenuwien & Allis, 2001). Modifications of histones include acetylation, methylation, phosphorylation and ubiquitination which are controlled by the action of several chromatin remodelers (Tessarz & Kouzarides, 2014; J. Wang, Qiu, et al., 2018).

Histone modifiers are divided into three main groups as “writer”, “eraser” and “reader” proteins. “Writer” and “eraser” proteins work together with “reader” proteins. They determine the level, location as well as the specificity of the modifications (Biswas & Rao, 2018). “Writer” proteins are responsible from introduction of the modification to the specific residue on the N - terminus histone tail, while “eraser” proteins are responsible from their removal. “Reader” proteins contain specialized domains that can recognize the target residue and epigenetic

modifications on them (Biswas & Rao, 2018; Paranjpe & Veenstra, 2015; J. Wang, Qiu, et al., 2018). As a result of recognition, they can recruit nuclear signaling network components onto chromatin, that plays role in events such as chromatin remodeling, gene expression, DNA damage response, DNA replication and recombination (Musselman et al., 2012).

Lysine residues on N-terminus tail of H3 and H4 can be acetylated. Acetylation is performed by histone acetyl transferases (HATs), and they can be removed by the action of histone deacetylases (HDACs). Bromodomain containing complexes are involved in the recognition of acetylated domains for recruitment of either activator or repressor transcription factor complexes. Acetylated histones create an open-chromatin configuration therefore these regions are considered as transcriptionally active regions, however, depending on the recruited complex, they can also repress transcription (Handy et al., 2012).

Lysine and arginine residues located on N-terminus of histones can be methylated. Lysine residues can be found in unmethylated, mono-methylated (me1), di-methylated (me2) and tri-methylated (me3) status. Methylation is performed by lysine methyltransferases (KMTs) and they are removed by the activity of lysine demethylases (KDMs) (J. Wang, Qiu, et al., 2018). They are recognized by proteins which contain specific domains such as Tudor domain, or chromodomain (Yap & Zhou, 2011). Histone methylation can have either gene silencing or gene activating effects according to the methylated residue and the level of the methylation. For example, while histone 3 lysine 9 trimethylation (H3K9me3), histone 3 lysine 27 trimethylation (H3K27me3) and histone 4 lysine 20 trimethylation (H4K20me3) are transcriptionally repressive modifications, histone 3 lysine 4 trimethylation (H3K4me3) which is generally found in promoter regions and histone 3 lysine 36 trimethylation (H3K36me3) that is generally found along the gene body are transcriptionally permissive modifications (Paranjpe & Veenstra, 2015).

ATP-dependent nucleosome remodeling complexes utilizes adenosine triphosphate (ATP) as an energy source to mediate movement of nucleosomes along the DNA (nucleosome rearrangement) as well as for their removal or exchange.

These complexes recognize the introduced histone marks and alter the chromatin accessibility to enable the activity of DNA binding proteins or histone binding proteins (Hargreaves & Crabtree, 2011; Tang et al., 2010). There are four subfamilies of ATP-dependent nucleosome remodeling complexes which are chromodomain-helicase DNA-binding (CHD), INO80, imitation switch (ISWI) and switch/sucrose non-fermentable (SWI/SNF). Presence of diverse specialized proteins in these complexes enable specific interactions with histone modifications as well as transcriptional activators or repressors to mediate their remodeling function (Clapier et al., 2017).

Combination of several modifications and the action of ATP-dependent complexes can have different effects on the chromatin state therefore, on the gene expression status.

1.3.1 SET Domain Containing Proteins and SETD3 Histone Methyltransferase

KMTs perform methylation either with the function of seven-beta-strand (7βS) domain or the SET domain (Husmann & Gozani, 2019). SET domain is named after three proteins that are identified in *Drosophila melanogaster* containing this domain, which are Suppressor of variegation 3-9, Enhancer of zeste and Trithorax (Herz et al., 2013; Husmann & Gozani, 2019; Shu & Du, 2021). Later, many SET domain containing proteins were identified in other organisms including mammals. SET domain is a ~130 amino acid long, evolutionarily conserved catalytic domain (Shu & Du, 2021). By using the cofactor SAM, it mono-, di- or trimethylates its substrates (Handy et al., 2012; Shu & Du, 2021). These substrates can be either lysine residues on the histone tails or non-histone proteins such as tumor suppressor proteins or transcription factors (Herz et al., 2013). SET domain containing proteins, like every other epigenetic regulator, are members of chromatin remodeling complexes such as Polycomb group (PcG) and Trithorax group (TrxG) complexes.

Other proteins in the complex help them to regulate specificity, and with the catalysis function (Herz et al., 2013; Schuettengruber et al., 2017).

SETD3 histone methyltransferase enzyme which contains a SET domain is required for the differentiation of mESCs into mesendoderm (Terzi Cizmecioglu vd., 2020). In humans it has three isoforms an the most well characterized isoform is 594 amino acids long (Witecka et al., 2021). Near the N-terminus end of SETD3 protein, there is a SET methyltransferase domain and at the C-terminus there is a Rubisco-LSMT domain (Shu & Du, 2021; Witecka et al., 2021). The function of Rubisco-LSMT domain is recognition of a methyl-accepting protein and binding to it, while SET domain is responsible from the transfer of the methyl group (Witecka et al., 2021). Similar to other epigenetic factors both nuclear and cytoplasmic functions have been identified in various cell types. Even though SETD3 is found in the nucleus, there is no identified nuclear localization signal (NLS) or an identified DNA binding domain. In the cytoplasm, level of SETD3 protein is regulated by the activity of GSK3β and F-Box and WD Repeat Domain Containing 7β (FBXW7β). Phosphorylation of SETD3 by GSK3β is followed by its ubiquitination via FBXW7β which leads to its proteolysis (Cheng et al., 2017).

In the nucleus, SETD3 performs its functions through interacting with transcription factors (TFs) or by controlling the histone methylation status. As a SET domain containing protein, SETD3 methyltransferase is shown to dimethylate 36th lysine residue of histone 3 (H3K36me2) *in vitro* and also in mouse fibroblast cells that overexpress SETD3 (Eom et al., 2011; Kim et al., 2011). Additionally, it can dimethylate 4th lysine residue of histone 3 (H3K4me2). In a study conducted in muscle cells, it was observed that SETD3 can bind to the promoter region of myogenin together with MyoD and upregulate its expression. With this, it has been found to have a role in muscle differentiation (Eom et al., 2011; Zhao et al., 2019). In different cell types, SETD3 functions by binding to various TFs and/or by methylating them. For example, it methylates FoxM1 to control the expression of VEGF (Cohn et al., 2016; Jiang et al., 2018). In colon cancer cells, in the presence

of DNA damage, it interacts with p53 protein to activate the expression of p53 target genes which leads to cell death through apoptosis (Abaev-Schneiderman et al., 2019). In another study performed using a cDNA library created using human muscle cells, it was shown that SETD3 has a relationship with the PCNA protein which is involved in cell division (Cooper et al., 2015). In liver cells, the increase in SETD3 levels causes an increase in cell division and enhances tumor formation (Cheng et al., 2017). Recently SETD3 was found to have a cytoplasmic function as an actin specific histidine N-methyltransferase (Kwiatkowski et al., 2018; Wilkinson et al., 2019). In vivo studies performed with SETD3 knockout human HAP1 cells or SETD3 knockout *Drosophila melanogaster* showed an absence of Histidine 73 (His73) methylation of β-actin when compared to wild type cells, indicating the role of SETD3 protein as an actin specific histidine N-methyltransferase (Kwiatkowski et al., 2018). Model organisms such as SETD3 knockdown and knockout fruit fly (*Drosophila melanogaster)* or SETD3 deleted mouse (*Mus Musculus)* have been produced and it has been observed that these organisms successfully reach to adulthood (Tiebe et al., 2018; Wilkinson et al., 2019). Deletion of the SETD3 gene from fruit flies did not affect traits such as fertility, hunger response, wing length, body weight, spawning, pupation, fat storage, climbing ability and glycogen levels. However, as a result of microarray analysis performed with SETD3 gene knockout fruit flies, it was found that expression of many genes was changed and most of these genes were related to muscle structure or contraction (Tiebe et al., 2018). These results observed in fruit flies are consistent with the results obtained from C2C12 cells, a myoblast cell line (Eom et al., 2011).

1.3.2 Epigenetic Regulations in Embryonic Stem Cells and During Differentiation

As embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) enable modeling various biological events including cell fate determination, they have an essential role in improvement of medicine. Epigenetic mechanisms plays a critical role in cell fate stability as well as regulations during differentiation processes (Terzi Cizmecioglu et al., 2020; H. Wu & Sun, 2006). A defect in epigenetic regulation at the ESC state can cause problems during differentiation processes. To maintain pluripotency in ESCs, several genes such as Oct4, Sox2 and Nanog should be expressed and signaling pathways should be tightly regulated (Descalzo et al., 2013; Morey et al., 2015; H. Wu & Sun, 2006). During differentiation of ESCs gene expression profiles change in a fast and complex manner. The regulation of these changes is critical for proper differentiation to take place. Epigenetic mechanisms such as DNA methylation and histone modifications are important during the differentiation processes as well as in the maintenance of pluripotency (H. Wu & Sun, 2006). For example, Oct4 locus, which is essential for pluripotency, is decorated with histone modifications that correlate with active transcription when cells are in the ESC state. As differentiation takes place, the locus becomes deactivated by the help of repressive modifications such as deacetylation, H3K9 methylation and promoter DNA methylation (G. Shi & Jin, 2010). During differentiation, Nanog, another gene involved in maintaining pluripotency, is also suppressed by promoter DNA methylation (Boland et al., 2014). As differentiation takes place repressive epigenetic modifications on lineage specific genes are removed and permissive histone marks comes into play. Overall, during differentiation dynamic epigenetic regulations change the accessibility of the chromatin, leading to expression of differentiation-specific genes and repression of pluripotency genes, thus a change in the cell character (Atlasi & Stunnenberg, 2017).

1.4 Cellular Signaling Pathways for Regulation of Pluripotency and Differentiation

Gene expression changes that result from epigenetic changes occur during ESC maintenance and differentiation. They are regulated by intracellular and extracellular signaling pathways. While some signaling pathways are only involved in maintenance of pluripotency, some pathways are also involved in differentiation processes. For example, PI3K/AKT and JAK/STAT3 signaling pathways which are activated by the LIF ligand are examples of pathways that help ESCs to maintain a pluripotent state (Ohtsuka et al., 2015). On the other hand, signaling pathways such as TGFβ/SMAD, MEK/ERK and canonical Wnt signaling pathway both plays a role in pluripotency maintenance at the ESC state and differentiation processes (Ma et al., 2016; Shoni et al., 2014).

1.5 Wnt Signaling Pathway

Wnt signaling pathway is mainly divided into two as non-canonical Wnt signaling pathways (β-catenin-independent pathway) and canonical Wnt signaling pathway (Wnt/β-catenin signaling pathway, β-catenin-dependent signaling pathway). Non-canonical Wnt signaling pathway is further subdivided into Wnt/Ca^{2+} pathway and Planar Cell Polarity Pathway (PCP). As studies on Wnt signaling pathways continue, different branches keep emerging (Komiya & Habas, 2008).

While non-canonical Wnt signaling pathways are not dependent on beta-catenin (β-catenin), it is the key transcription factor of the canonical Wnt signaling pathway (Komiya & Habas, 2008). Non-canonical Wnt signaling pathways inhibit the canonical Wnt signaling pathway through various ways. Activation of Wnt/Ca^{2+} pathway, inhibits the canonical Wnt signaling pathway through Nemo-like kinase (NLK) (Ackers & Malgor, 2018). Other inhibition mechanisms involve increased β-catenin destruction, independent from GSK3β mechanism and increased expression of canonical Wnt signaling pathway inhibitors such as Wnt5a, Wnt5b or Dickkopf

Wnt Signaling Pathway Inhibitor 1 (DKK1) (Amerongen & Nusse, 2009; Topol et al., 2003).

Wnt signaling pathways are activated by hydrophobic, insoluble glycoproteins called Wnt ligands. In mammals 19 Wnt ligands are present and their expression is cell or tissue type specific (Voloshanenko et al., 2018). After being synthesized in the cell, Wnt ligands, are palmitoylated for secretion and glycosylated for proper function. They are transported out of the cell to bind their target receptors and activate the target Wnt signaling pathways (Ackers & Malgor, 2018). For example, Wnt3a ligand activates the canonical Wnt signaling pathway by binding to the Frizzled (Fzd) receptor together with LRP5 or LRP6 co-receptor. Wnt1, Wnt2, Wnt8a, Wnt8b, Wnt10a and Wnt10b are other ligands that are known to activate the canonical Wnt signaling pathway (Ackers & Malgor, 2018; Grumolato et al., 2010) On the other hand, Wnt5a ligand activates the non-canonical Wnt signaling pathway by binding to Fzd receptor together with Ror1 or Ror2 co-receptor complexes (Grumolato et al., 2010). Wnt5b, Wnt6, Wnt7a and Wnt7b are other ligands that activate the non-canonical Wnt signaling pathway. Even though there was a classification of Wnt ligands as canonical and non-canonical Wnt signaling pathway activating ligands, recent findings showed that depending on unique receptor - coreceptor combinations, same Wnt ligand can activate either canonical or non-canonical Wnt signaling pathway. (Ackers & Malgor, 2018; De, 2011). For example, Wnt11 and Wnt4 were normally considered as non-canonical Wnt pathway activating ligands, however recent research revealed that they are also able to activate the canonical Wnt signaling pathway (Komiya & Habas, 2008; Q. Zhang et al., 2021). Therefore, presence of different receptor – coreceptor complexes might result in different downstream effects.

1.5.1 Non-canonical Wnt Signaling Pathways (β-catenin-independent Pathways)

Planar Cell Polarity (PCP) pathway, a branch of non-canonical Wnt pathway, is activated by Wnt5a, Wnt5b or Wnt 11 ligands (Boligala et al., 2022; D.-L. Shi, 2022). It mainly plays a role in modification of the cell cytoskeleton (Komiya & Habas, 2008). It regulates polarization of cells, the migration of dorsal mesodermal cells during convergent extension of gastrulation and the migration of cell during neural tube closure (D.-L. Shi, 2022; Veeman et al., 2003; Wallingford et al., 2002).

Wnt/Ca^{2+} signaling pathway, another branch of the non-canonical Wnt pathway, is activated by Wnt ligands such as Wnt5a and Wnt11 (Voloshanenko et al., 2018). Activation of the pathway causes calcium release, a secondary messenger, into the cell. This calcium wave is thought to be involved in early pattern formation as well as heart formation during gastrulation (Kohn & Moon, 2005; Slusarski & Pelegri, 2007). Activation of this pathway also influences cell adhesion and migration (Boligala et al., 2022). Additionally, it regulates the canonical Wnt signaling pathway, which plays a role in dorsal axis formation, and the PCP signaling pathway that is involved in gastrulation (Komiya & Habas, 2008).

For detailed representations non-canonical Wnt pathways, *see* Appendices D.

1.5.2 Canonical Wnt Signaling Pathway (Wnt/β-catenin Signaling Pathway, β-catenin-dependent Signaling Pathway)

The canonical Wnt signaling pathway is activated by Wnt ligands such as Wnt3a (Hayat et al., 2022). When the pathway is not active, a protein complex called the “destruction complex” which is composed of Axin, adenomosis polyposis coli (APC), protein phosphatase 2A (PP2A), casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β) actively phosphorylates N-terminal Ser33, Ser 37, Ser45 and Thr41 residues of cytoplasmic β-catenin (Boligala et al., 2022; Hagen & Vidal-Puig, 2002; C. Liu et al., 2002; Miki et al., 2011). These phosphorylation

events lead to its ubiquitination by β-transducing repeat-containing E3 ubiquitin-protein ligase (β-TrCP) followed by its degradation through the action of 26S proteasome (Azzolin et al., 2014; Boligala et al., 2022; Valenta et al., 2012).

As canonical Wnt signaling pathway is activated, cytoplasmic Dishevelled (Dvl) protein is recruited to the receptor complex through the action of Fzd receptors. Receptor bound Dvl, multimerizes and causes recruitment of kinases such as CK1α and GSK3β, which are components of the "destruction complex" (Valenta et al., 2012). This translocation prevents destruction of β-catenin and leads to its stabilization. As a transcription factor, stabilized β-catenin translocates into the nucleus to perform its function which is to regulate transcription of its target genes (Clevers, 2006; Voloshanenko et al., 2018).

β-catenin is found both in the plasma membrane, in the cytoplasm and in the nucleus. Extracellular signals such as Wnt ligands regulate the changes in its cellular localization. In mammals, a pool of cytoplasmic β-catenin binds to E-cadherin to form the adherens junctions and mediate the interaction of E-cadherin and actin cytoskeleton (Hülsken et al., 1994). As adherens junctions are involved in cell-to-cell contact through interaction of E-cadherin molecules of the two cells, β-catenin which is bound to E-cadherin is also located in the plasma membrane (Hülsken et al., 1994; Valenta et al., 2012). In the nucleus, it acts as a transcription factor, downstream of the canonical Wnt signaling pathway (Clevers, 2006).

β-catenin is a member of Armadillo (ARM) repeat protein superfamily. Human β-catenin is composed of 781 amino acids. In the central region it contains 12 imperfect ARM repeats each consisting of ~42 amino acids (Xing et al., 2008). All ARM repeats fold onto each other to generate a superhelix which has a positively charged groove. Proximal to its C-terminus, next to the 12th ARM repeat, β-catenin has a conserved, specific helix called Helix-C. Helix-C was shown to be important for transcriptional activity of β-catenin as it is found to be interacting with C-Terminal Transcriptional Activators (CTTA), but it is not for the cell-cell adhesion function (Valenta et al., 2012). Structures of N-terminal domain (NTD) and C-

terminal domain (CTD) are flexible; however, central region is relatively more rigid, and it generates a scaffold that enables interaction with β-catenin binding partners (Huber et al., 1997; Valenta et al., 2012). It is suggested that NTD and CTD also folds back onto the central region to enable binding to proteins such as T-cell factor / lymphoid enhancer factor (TCF/LEF) (Valenta et al., 2012). Biochemical analysis suggests that β-catenin function in a monomeric form for its transcriptional activity and it dimerizes with α-catenin to form adherens junctions (Gottardi & Gumbiner, 2004). β-catenin binding proteins, such as APC, E-cadherin or TCF/LEF all interact with R3-R9 ARM repeats by forming salt bridges with Lysine 312 (Lys312) and Lysine 435 (Lys435) residues. Rest of the ARM repeats are involved in strengthening the interaction. As β-catenin binding proteins interact with the same residues of the central region groove, binding of these proteins to β-catenin is not simultaneous (Huber & Weis, 2001; Spink et al., 2001; Valenta et al., 2012).

Even though β-catenin functions in the nucleus as the key transcription factor of the canonical Wnt signaling pathway, it does not have a nuclear localization signal (NLS) or a nuclear export signal (NES). While how β-catenin enters to the nucleus is not clear, it was shown that this process is independent of importin-karyopterin (Fagotto et al., 1998). Additionally, β-catenin was shown to interact with components of nuclear pore complex even though import and export mechanism is not clear (Shitashige et al., 2008; Valenta et al., 2012). In mammals, FoxM1 transcription factor was also found to promote nuclear import of β-catenin by binding to its 11th and 12th ARM repeats (N. Zhang et al., 2011). Interaction of β-catenin and FoxM1 is further maintained in the nucleus suggesting a β-catenin nuclear anchoring function of FoxM1. In the nucleus, it was found to be bound to the promoters of Wnt target genes in a complex with β-catenin and TCF (Valenta et al., 2012). Moreover, posttranslational modifications of β-catenin are suggested to be involved in its nuclear import, as phosphorylation of its 191st and 605th residues by JNK2 was shown to lead its nuclear transport, however this requires further confirmation (X. Wu et al., 2008).

β-catenin does not have a DNA binding domain, therefore, this function is mediated by its nuclear binding partners such as FoxM1, proteins from Foxo, Smad and nuclear receptor families or transcription factors such as TCF/LEF (Miki et al., 2011; Pagella et al., 2022; Valenta et al., 2012; N. Zhang et al., 2011). When β-catenin is not in the nucleus, TCF is located on the target DNA loci, containing the Wnt responsive elements (WREs). There, it is also in a complex with Groucho/transducin-like Enhancer of split (GRG/TLE) proteins, and it functions as a transcriptional repressor. As stabilized β-catenin translocates into the nucleus, it binds to the N-terminus of TCF/LEF transcription factors and physically displaces GRG/TLE. This displacement converts TCF into a transcriptional activator and promotes the transcription of target genes which also include positive and negative regulators of the pathway (Clevers, 2006; Valenta et al., 2012). Additional to TCF/LEF/β-catenin interaction, β-catenin also interacts with co-activators like CBP and p300, histone acetylases, and MLL1, histone methyltransferase to regulate transcriptional activation (Miki et al., 2011; Pagella et al., 2022; Sierra et al., 2006). Some of the β-catenin/TCF complex target genes are cMyc, CyclinD1, Axin2, LRP, Fzd, TCF/LEF, c-jun, Dkk1 and Twist (Clevers, 2006; Lecarpentier et al., 2019; Maretzky et al., 2005; Reiss et al., 2005; Valenta et al., 2012).

Besides transcriptional activation, β-catenin can also cause transcriptional repression when bound to promoter regions of several genes (Pagella et al., 2022; Valenta et al., 2012). For example, in keratinocytes binding of β-catenin to the promoter region of E-cadherin represses its transcription (Jamora et al., 2003). $p16^{INK4a}$ gene in melanocytes is another gene which is repressed when promoter region is bound by the TCF/β-catenin complex (Delmas et al., 2007).

Canonical Wnt signaling pathway is involved in many cellular processes during different stages of development and homeostasis (Pagella et al., 2022). Deregulation of it causes several diseases including Alzheimer’s disease, cancer, heart disease and osteoarthritis (Shah & Kazi, 2022). It also plays various roles during ESC state, during differentiation as well as in adult tissues (Valenta et al., 2012). Depending on the variable expression of Wnt ligands, Wnt pathway related

receptors and/or the secreted inhibitors, the roles of canonical Wnt signaling pathway changes during ESC state and during differentiation (Amerongen & Nusse, 2009; Hoppler & Kavanagh, 2007; MacDonald et al., 2009).

For detailed representation of canonical Wnt pathway, *see* Appendices D.

1.6 Role of Canonical Wnt Signaling Pathway at ESC Stage

mESCs actively produce Wnt ligands. It has been observed that they lose their self-renewal ability as the production of Wnt ligands is blocked. In addition, when β-catenin is stabilized by GSK3 inhibitors such as CHIR99021, 6-bromoindirubin-3'-oxime (BIO) or lithium chloride, both mouse and human ESCs can be kept in a self-renewing state. Wnt/β-catenin signaling pathway is also shown to maintain self-renewal ability of neural or hematopoietic stem cells (Miki et al., 2011). Additionally, canonical Wnt signaling pathway is shown to maintain self-renewal ability of neural or hematopoietic stem cells (Miki et al., 2011). These observations indicate that the Wnt/β-catenin signaling pathway has an important role in stem cell regeneration (Merrill, 2012; Ogawa et al., 2006; Takao et al., 2007). However, there is still a contradiction about the effect of β-catenin on pluripotency maintenance as different studies obtained controversial results (Muñoz-Descalzo et al., 2015).

1.7 Role of Canonical Wnt Signaling Pathway During Differentiation

In human embryonic stem cells (hESCs) and in mouse epiblast stem cells (EpiSCs) which resemble to each other, it is observed that β-catenin requirement is low in ESC maintenance but there is a clear requirement of transcriptional activity of β-catenin for proper differentiation. Therefore, they suggested that the role of β-catenin in ESCs and EpiSCs is to balance self-renewal and differentiation; it is involved in promoting self-renewal when found in a protein complex, and it promotes differentiation through its transcriptional activity (Muñoz-Descalzo et al.,

2015). In hESCs, canonical Wnt signaling pathway was found to be regulating genes that are associated with pluripotency exit and genes that control the commitment towards mesodermal lineage (Pagella et al., 2022). Several groups reported that Wnt signaling pathway activity in mESCs induce endoderm and mesoderm differentiation, as activation of the pathway induced high expression of endodermal and mesodermal markers like AFP, Brachyury (T), Foxa2, Flk-1 and Lhx1 (Miki et al., 2011). Mesoderm is the key layer that participates in axis formation, therefore, canonical Wnt signaling pathway also has an important role in axis formation in early embryos (Arnold & Robertson, 2009). When ctnnb1Δ mESCs (β-catenin knockout) were used, it was observed that gastrulation process could not occur, which proves the importance of β-catenin in this process (Merrill, 2012). Additionally, Wnt/β-catenin signaling pathway is involved in determining the anteroposterior (AP) body axis during early embryonic development, in the formation of primitive streak that originates from mesendoderm and in organ formation (Amerongen & Nusse, 2009; Barker & van den Born, 2008; Berge et al., 2008; Clevers, 2006; Merrill, 2012; Mohamed et al., 2004; Sokol, 2011). When β-catenin is genetically inactivated in mice, defects were observed in formation of AP axis, definitive endoderm, mesoderm and ectoderm (Lyashenko et al., 2011). Results of experiments performed on mouse and frog embryos showed that the canonical Wnt signaling pathway inhibits the neural development and ensures mesendoderm differentiation (Bakre et al., 2007; Gadue et al., 2006; Haegele et al., 2003; Heeg-truesdell & Labonne, 2006; Itoh & Sokol, 1999; Lindsley et al., 2006; Sokol, 1993; Takada et al., 1993; Yoshikawa et al., 1997). In mouse embryos that lack Wnt3, LRP5/6 and β-catenin, proper body axis formation (AP axis formation) was not observed and anterior neuroectoderm formation was excessive (Haegel et al., 1995; Huelsken et al., 2000; Kelly et al., 2004; P. Liu et al., 1999).

Epithelial-to-mesenchymal transition (EMT) is an important developmental event that occurs during gastrulation and canonical Wnt signaling pathway is one of the pathways that is responsible from its regulation. During gastrulation, embryonic epithelium undergoes EMT to generate mesodermal cells. Immobile epithelial cells

turn into fibroblastoid mesenchymal cells which are highly mobile (Thiery et al., 2009). During this process, E-cadherins disappear from the cell membrane and N-cadherins takes their place (Zeisberg & Neilson, 2009). Canonical Wnt signaling controls expression of transcription factors which regulate E-cadherin expression. Additionally, fates of other epithelial molecules are controlled by the direct and indirect transcriptional targets of the canonical Wnt signaling pathway. For example, Twist, one of these TFs, is an important factor defining the mesoderm in both vertebrates and invertebrates (Howe et al., 2003; Zeisberg & Neilson, 2009). Action of Twist directly causes downregulation of E-cadherin expression, while upregulating the expression of fibronectin and N-cadherin, leading to EMT (J. Yang et al., 2004; Z. Yang et al., 2007). Additional to Twist, Snail 1 and Snail2 are other identified factors which are controlled by the canonical Wnt signaling pathway and involved in regulation of EMT by repressing E-cadherin transcription (Barrallo-Gimeno & Nieto, 2005; Conacci-Sorrell et al., 2003). ZEB1 transcription factor also controls EMT, and it is a direct target of the canonical Wnt signaling pathway. It leads to transcriptional repression of E-cadherin, and activation of mesenchymal genes (Sánchez-tilló et al., 2011).

1.8 Preliminary Data

In the previous study, in which epigenetic factors that regulate mesendoderm differentiation were determined, it was observed that mESCs whose SETD3 expression is suppressed with shRNA could not differentiate into mesoderm or endoderm (Terzi Cizmecioglu vd., 2020). This result is further confirmed with Setd3 deleted (setd3Δ) mESCs, which were created with CRISPR-Cas9 technique, grown in standard serum-containing mESC medium (*see* Figure 1.1). *Brachyury* expression in wild type mouse cells (wild type cells in CJ9 background), which is an early endoderm and mesoderm marker (Faial et al., 2015), dramatically increases at the 5th day of mesoderm and endoderm differentiation. In setd3Δ cells, this increase is not observed (*see* Figure1.1a, Figure 1.1b). Expression of *Foxa2*, which is an early

marker of endoderm (Faial et al., 2015), increases dramatically in wild type cells at the 5th day of endoderm differentiation, however, this increase is not observed in setd3Δ cells (*see* Figure 1.1c). When both wild type and setd3Δ cells were differentiated towards neuroectoderm, Sox1 and Pax6 which are neuroectoderm markers were observed to be similar (*see* Figure 1.1d and e). Additionally, levels of pluripotency markers, Oct4 and Nanog, were also observed to be similar between wild type and setd3Δ cells both at the mESC state and the 4th and 5th days of endoderm and mesoderm differentiation (*see* Figure 1.1f and g). This suggests that when mESCs grown in standard serum-containing mESC medium are differentiated towards endoderm or mesoderm, absence of SETD3 is causing a differentiation defect, while differentiation towards neuroectoderm and expression of pluripotency markers are not dramatically altered.

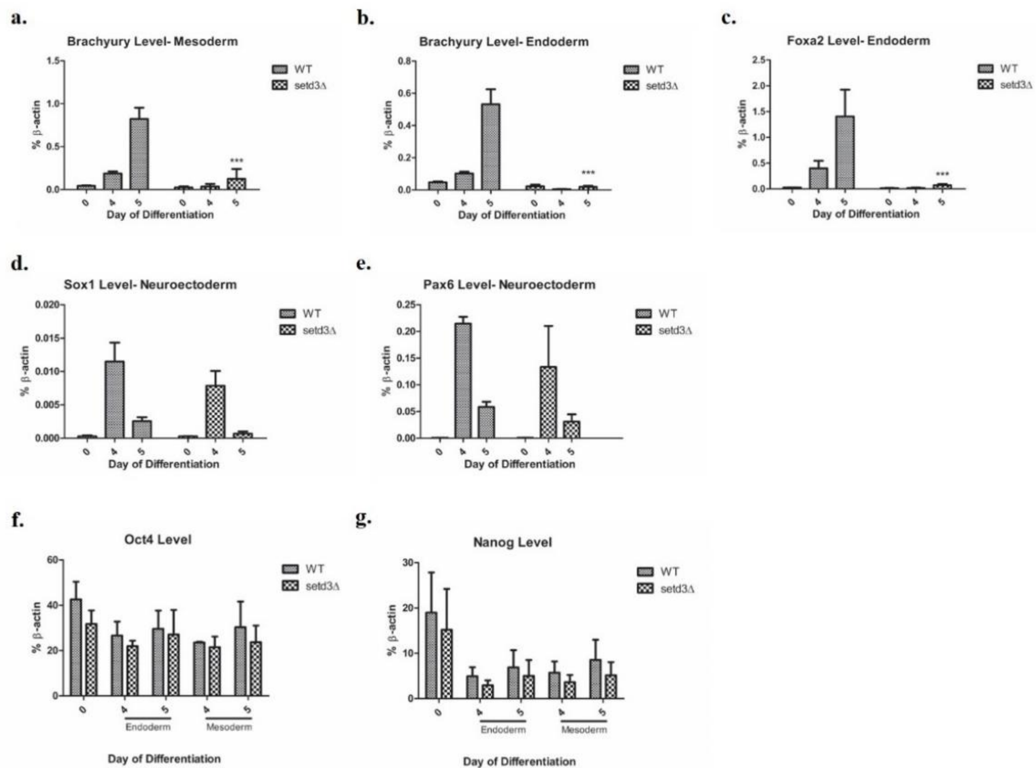

Figure 1.1. Presence of SETD3 in mESCs is important for differentiation towards mesoderm and endoderm. RT-qPCR analysis of wild type (WT) and setd3Δ cells, grown in standard serum-containing mESC medium, at the mESC state and at the 4th and 5th days of differentiation towards endoderm, mesoderm or neuroectoderm. **A.** Expression level of *Brachyury* (*Bry*) during mesoderm differentiation. **B-C.** *Bry* and *Foxa2* expression levels

during endoderm differentiation. **D-E.** *Sox1* and *Pax6* expression levels during neuroectoderm differentiation. **F-G.** *Oct4* and *Nanog* levels in ESCs state and during 4th and 5th days of endoderm and mesoderm differentiation. Expression levels are given as a percentage of β-actin expression. Graphs are representative of at least 3 replicates. Statistical analysis (t-test or two-way ANOVA) was performed using GraphPad Prism Software. Error bars indicate ± SEM. ***: p-value < 0.001.

The mechanism by which SETD3 controls mESC differentiation is unclear. Preliminary data from our TUBITAK 1001 project (119Z405) suggests that setd3Δ mESCs grown in standard serum-containing mESC medium fail to complete differentiation process in the time course of wild type mESC differentiation. They begin to differentiate later, and the process is slower. In the same project, an RNA-seq library was generated using RNA samples of wild type and setd3Δ cells which are collected from the mESC state as well as from 2nd, 3rd and 4th days of endoderm differentiation. By performing KEGG analysis using differentially expressed genes (DEGs) between these two cell lines, significantly enriched pathways were identified. It has been observed that Wnt signaling pathway is enriched by genes that are expressed more in wild type cells when compared setd3Δ cells (up regulated), at the mESC state and at the 2nd and 3rd days of endoderm differentiation (*see* Figure 1.2). This result suggests that endoderm differentiation defect observed in setd3Δ mESCs might be due to the inability of these cells to activate the Wnt signaling pathway.

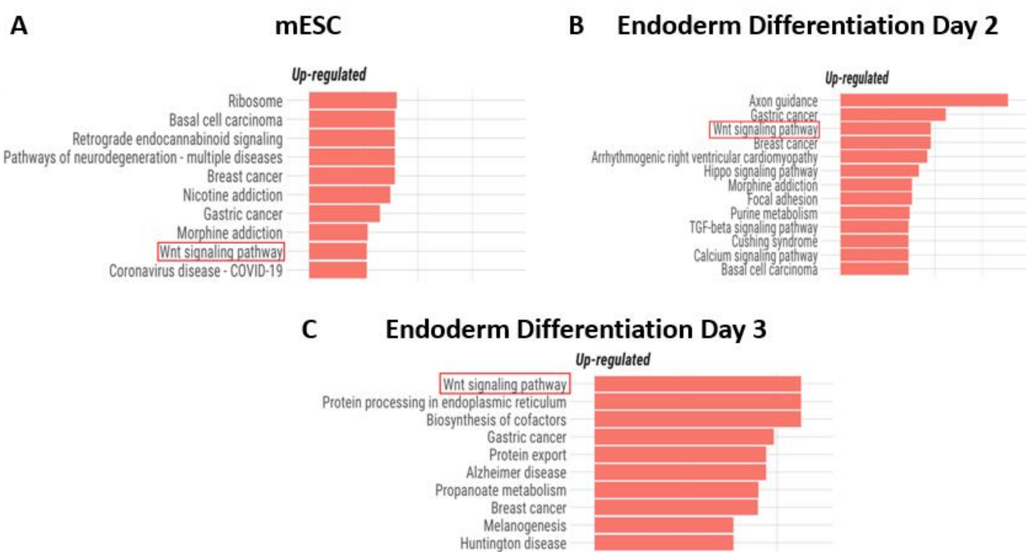

Figure 1.2. At the mESC state as well as during 2nd and 3rd days of endoderm differentiation, Wnt pathway related genes are expressed significantly more in wild type cells than setd3Δ cells which were grown in standard serum-containing mESC medium at the mESC state. According to gene expression levels, most significantly enriched, up-regulated pathways between wild type and setd3Δ cells (wild type cells / setd3Δ cells) are shown in **A.** mESC state, **B.** 2nd day of endoderm differentiation, and **C.** 3rd day of endoderm differentiation. mESCs were grown in standard serum-containing mESC medium prior to differentiation. RNA samples were collected from mESCs, 2nd and 3rd days of differentiation and an RNA-seq library was produced. Pathway analysis was performed on KEGG database.

As described in section 1.2, cells can be cultured using two different techniques. Either on a feeder layer, in standard serum-containing mESC medium, or without a feeder layer, in 2i4 medium which contains low percentage of serum (4%), LIF as well as two inhibitors, GSK3 inhibitor (CHIR99021) and MEK1/2 inhibitor (PD335901). When, endoderm differentiation experiment was repeated using mESCs grown in 2i4 medium, it was observed that setd3Δ mESCs were able to differentiate into endoderm (*see* Figure 1.3). In wild type cells, levels of endoderm markers, Bry and Foxa2, reached a high level starting from the 4th day of differentiation. These levels indicate endoderm differentiation is successful. Although not exactly at the same levels, setd3Δ cells also showed an increase in the expression of these markers at the 3rd day of differentiation. This result is consistent with the prediction that endoderm differentiation defect observed in setd3Δ cells might be due to inability of these cells to activate the Wnt signaling pathway as GSK3 inhibitor in this medium activates the canonical Wnt signaling pathway by preventing destruction of β-catenin. Normal differentiation time course observed in setd3Δ mESCs grown in 2i4 medium might be the result of stimulated expression of Wnt target genes which reached similar expression levels to wild type cells upon GSK3 inhibitor stimulation.

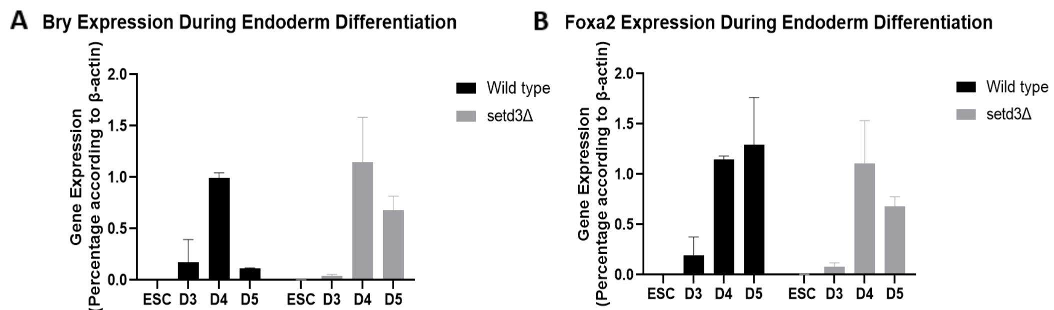

Figure 1.3. When setd3Δ mESCs were grown in 2i4 medium at the mESC state, normal endoderm differentiation is observed. RT-qPCR analysis of wild type and setd3Δ cells, grown in 2i4 medium, at the mESC state and at the 3rd, 4th and 5th days of endoderm differentiation. Expression levels of endoderm markers **A.** Bry and **B.** Foxa2. Expression levels are given as a percentage of β-actin expression. Graphs are representative of 2 replicates and generated using GraphPad Prism Software. Error bars indicate ± SEM.

From previous studies it is known that medium that mESCs are cultured in can affect their potency, the ability of them to differentiate into variety of specialized cell types (Gonzalez et al., 2016). The difference between Figure 1.1 and Figure 1.3 is the medium mESCs were grown in, and this indicates that the medium cells were cultured during mESC state affected their ability to differentiate into endoderm at a later time. This observation is important to notice that endoderm differentiation defect observed in setd3Δ mESCs is due to the ESC state and it can be reversed by culturing mESCs in 2i4 medium. Therefore, future studies on the role of SETD3 in mESC differentiation are focused on the ESC state, where cells have not yet lost their pluripotency.

Additional support for a possible role of SETD3 on Wnt pathway regulation came from the SETD3 interactome in mESCs. Previously, two replicates of nuclear streptavidin-based immunoprecipitation followed by mass spectrometry (IP-MS) was performed in our laboratory (TUBITAK 3501 project, 117Z953). SETD3 was found to interact with some Wnt signaling pathway related proteins, although not with β-catenin itself (*see* Table 1). Transcription factor E2-alpha (TCF3 or TFE2), CREB Binding Protein (CREBBP), B-cell CLL/lymphoma 9 protein (BCL9), Pygopus Family PHD Finger 2 (PYGO2) and Casein kinase I isoform delta (CSNK1D) are SETD3 interacting proteins that are involved in the canonical Wnt signaling pathway and Rho-associated protein kinase 2 (ROCK2) is a SETD3 interacting protein which is unique to PCP pathway, a part of non-canonical Wnt pathway (Cruciat, 2014; Van Andel et al., 2019).

Table 1. SETD3 is interacting with proteins that are involved in Wnt signaling pathways.

Protein Name	*Gene Symbol*	*UniProt ID*	*Wnt Signaling Pathway*
Transcription factor E2-alpha	Tcf3	P15806	Canonical
Histone lysine acetyltransferase CREBBP	Crebbp	P45481	Canonical
B-cell CLL/lymphoma 9 protein	Bcl9	Q9D219	Canonical
Pygopus	Pygo2	Q3V113	Canonical
Casein kinase I isoform delta	Csnk1d	Q9DC28	Canonical
Rho-associated protein kinase	Rock2	P70336	Non-canonical

Among these interacting proteins, TCF3 is found to be repressing various genes in stem cells as well as vertebrate embryos (Hikasa et al., 2010). It was found to be associated with promoters of pluripotency factors to repress their activity. Action of canonical Wnt signaling pathway is proposed to support pluripotency by either converting TCF3 associated complexes from repressors to activators, or by removing TCF3 and leading to recruitment of other TCF factors which activates transcription through interacting with β-catenin (Cole et al., 2008; Wray et al., 2011). SETD3 can also perform its function by dimerizing with MyoD to promote expression of myogenin (Eom et al., 2011). MyoD was also found to be interacting with TCF3 (Chakraborty et al., 1992; Langlands et al., 1997; Maleki et al., 1997; Patel & Chaudhary, 2012). Since SETD3 has been shown to be involved in muscle differentiation through its interaction with MyoD, TCF3-SETD3 interaction might be important for ESC differentiation (Eom et al., 2011).

In different experiments SETD3 and TCF3 is also found to be interacting with CREBBP, which is known as CBP (Bradney et al., 2002). CREBBP is one of

the co-activators of β-catenin which performs histone acetylation leading to expression of canonical Wnt signaling pathway target genes (Valenta et al., 2012).

BCL9 and PYGO2 are other co-activators of β-catenin which are also found to be interacting with SETD3. BCL9 is a β-catenin specific co-activator. It binds to both β-catenin and PYGO2, performing a bridge function to bring these proteins together. Interaction of these three proteins is shown to be important for the maximal canonical Wnt signaling pathway output in mammalian cells (Vafaizadeh et al., 2021). In mammals, BCL9 is also found to be interacting with other β-catenin co-activators such as CBP/p300 via its C-terminus (Sustmann et al., 2008). In MCF10A cell line, which is a non-malignant breast cell line, PYGO2 was found to be globally facilitating histone 3 lysine 4 trimethylation (H3K4me3), including canonical Wnt signaling pathway target gene loci, through its "reader" function. Global increase in H3K4me3 was shown to be the result of WDR5 recruitment, which is a SET domain containing histone methyltransferase. However, even though lack of PYGO2 changed the levels of H3K4me3 modification at canonical Wnt signaling target loci, interaction of PYGO2 and WDR5 was also detected in the absence of extracellular Wnt signaling like stimulation (Gu et al., 2009).

CSNK1D which is also known as CK1δ, is an isoform of CK1α which plays a role in "destruction complex" and leads to degradation of β-catenin. In 2004, CSNK1D was found to be a positive regulator of the canonical Wnt signaling pathway (Swiatek et al., 2004). The observation that CSNK1D was also found to be phosphorylating Lrp6 co-receptor leading to Axin recruitment and β-catenin stabilization supports this finding (McCubrey et al., 2014). However, in 2005, it was found to inhibit target gene expression of canonical Wnt signaling pathway by phosphorylation LEF1 and causing disassembly of β-catenin/LEF1 complex from the target gene promoters (Schittek & Sinnberg, 2014). Additionally, CSNK1D was found to be phosphorylating APC, a component of the "destruction complex" and enhancing its affinity to β-catenin leading to its degradation (Cruciat, 2014). According to different external stimuli these functions of CSNK1D might be coming into play.

TCF3, CREBBP and BCL9 which are members of the canonical Wnt signaling pathway and are directly interacting with β-catenin. Additionally, they were found to be interacting with SETD3 as a result of the nuclear mass spectrometry analysis. Another mutual interaction partner of SETD3 and β-catenin is FoxM1 transcription factor. Both of these proteins interact with FoxM1 and regulate transcription of target genes, VEGF expression is regulated when interacting with SETD3, and possibly canonical Wnt signaling target genes are regulated when it is in a complex with β-catenin and TCF as this complex was found to be bound to target gene promoters (Cohn et al., 2016; Jiang et al., 2018; N. Zhang et al., 2011). Additionally, co-activators of β-catenin also includes many histone modifiers and ATP-dependent nucleosome remodeling complexes. β-catenin co-activators are found to introduce ubiquitination which is followed by H3K4 trimethylation. H3K4 trimethylation is performed with the function of MLL1/MLL2 SET1-type HMT complex (Sierra et al., 2006). Furthermore, recent research reported that mono-methylation of H3K20 which is mediated by SET8 occurs upon binding to β-catenin. This modification was found to be important for β-catenin induced transcription (Li et al., 2011). PYGO2, a co-activator of β-catenin, was also shown to recruit a SET domain containing histone methyltransferase, WDR5 (Gu et al., 2009). Overall, these suggest that SETD3, a SET domain containing protein, which introduces activatory modifications like H3K36me2 and H3K4me2 might be interacting with β-catenin by itself, or in a protein complex to activate the transcription of β-catenin target genes. Therefore, this interaction might play a role in the activity of canonical Wnt signaling pathway. In the absence of SETD3, this activation might not be sufficiently achieved which results with the observed differentiation defect in setd3Δ cells grown in standard serum-containing mESC medium (*see* Figure 1.2). The finding that transcriptional activity of β-catenin was found to be important for differentiation further supports this prediction.

1.9 Aim of the study

mESCs are pluripotent cells that can form three primitive layers: endoderm, mesoderm and neuroectoderm. We have observed endoderm differentiation defect in the absence of SETD3 methyltransferase and wanted to elucidate the function of it during this process. According to our preliminary data, we decided to focus on its effect on the canonical Wnt signaling pathway. In this project, we hypothesized that SETD3 protein regulates endoderm differentiation of ESCs by regulating the canonical Wnt signaling activity, and the differentiation defect observed in setd3Δ cells which are grown in standard serum-containing mESC medium is due to insufficient activation of the canonical Wnt signaling pathway. Our aim in this thesis was to determine the effect of SETD3 on β-catenin level and localization as well as the canonical Wnt pathway activity in mESCs grown in different medium formulations, either standard serum-containing mESC medium or 2i4 medium. Overall, we observed that setd3Δ mESCs grown in standard serum-containing mESC medium had lower levels of nuclear β-catenin and, regardless of the growth medium, mESCs that lack SETD3 showed significantly lower levels of canonical Wnt signaling pathway activity when compared to wild type cells grown in the same medium. These results suggest that presence of SETD3 is affecting the nuclear levels of β-catenin as well as the canonical Wnt signaling pathway activity. Therefore, lower canonical Wnt signaling pathway activity may have a role in the differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Mouse Embryonic Fibroblast (MEF) Culture and Mitomycin C Treatment

Primary BALB-c MEFs were acquired from Koç University. They were expanded in 15-cm gelatinized (0.1% gelatin solution, *see* Appendices B) tissue culture (TC) dishes in MEF medium (*see* Appendices A) at 37°C, 5% CO_2 TC incubator. As cells reached 80-90% confluency, they were passaged using Trypsin EDTA solution B (Cat. No.: BI03-052-1B, Biological Industries). To increase the growth capacity, between passages three and five, cells were grown in standard serum-containing mESC medium (*see* Appendices A). As they started to expand faster, MEF medium was used until passage seven. At passages seven or eight, to block the cell division process, Mitomycin C treatment was performed for 2 hours, using Mitomycin C lyophil. research grade (Cat. No.: SE2980501, Serva) containing MEF medium. After treatment, they are now called mitoMEFs.

2.1.2 mESC Culture

2.1.2.1 mESC Culture in 2i4 Medium

CJ9 mESCs (wild type, WT) and mESCs whose *Setd3* gene was deleted with CRISPR-Cas9 system (setd3Δ, knockout, KO) were acquired from Boston Children's Hospital from the laboratory of Prof. Stuart Orkin. Wild type and setd3Δ mESCs were grown in 0.1% gelatin coated 6-well TC plates in 2i4 medium (*see*

Appendices A) at 37°C, 5% CO_2 TC incubator. As cells reached 80-90% confluency, they were passaged using 1X TrypLE Express Enzyme (Cat. No.: 12605-010, Gibco).

2.1.2.2 mESC Culture in Standard Serum-Containing mESC Medium

Wild type and setd3Δ mESCs were grown in gelatinized 6-well TC plates on mitoMEFs in standard serum-containing mESC medium, inside 37°C, 5% CO_2 TC incubator. As cells reached 80-90% confluency, they were passaged using Trypsin EDTA solution B (Cat. No.: BI03-052-1B, Biological Industries). Before performing further experiments, mESCs were separated from mitoMEFs using Balbasi et al. protocol. As for western blotting experiments, mESCs that were separated from mitoMEFs were seeded to gelatinized 10-cm cell culture plates in 8 mL standard serum-containing mESC medium to make sure they divide again and outnumber the remaining mitoMEFs. After 24 hours they were collected for the experiment.

2.2 Phalloidin Staining

Before starting the experiment, 12 mm round coverslips (Cat. No.: 126.02.024, Isolab) were placed into 12-well TC plates and gelatinized using 0.1% gelatin solution. 1.5 x 10^5 live wild type mESCs, 1.5 x 10^5 live setd3Δ mESCs and 1.5 x 10^5 live setd3Δ + pFLBioSetd3 (Rescue) mESCs grown in standard serum-containing mESC medium were seeded onto coverslips. Rescue cell line was prepared within the scope of our previous TUBITAK 1001 project (119Z405). Media was completed to 1 mL by using standard serum-containing mESC medium. After around 24 hours coverslips were transferred into 10 cm petri dishes and a humidity chamber was generated using wet paper towels, to avoid drying of cells. To remove remaining media, coverslips were washed briefly with 250 µL 1X PBS. Later, PBS was removed, and cells were fixed with 250 µl 4% PFA (*see* Appendices B) for 20 minutes at room temperature (RT). After briefly washing the coverslips with 250 µl

1X PBS, 0.5% Triton X-100 (*see* Appendices B) was added onto coverslips to permeabilize the cells and incubated at RT for 10 minutes. Coverslips were washed with 0.1% Triton X-100, three times, for 5 minutes at RT. 1% BSA (*see* Appendices B) was incubated for 10 minutes at RT for blocking and 40 µL 1:200 diluted Alexa Fluor™ 488 Phalloidin stain (Cat. No.: A12379, Thermo Fisher Scientific) was added onto coverslips, incubated at RT for 45 minutes. Finally, coverslips were washed briefly with 0.1% Triton X-100 and DAPI containing mounting medium (Cat. No.: DUO82040, Sigma) was added on top of cells for mounting. Coverslips were placed onto slides as cells were facing down. The edges of the coverslips were sealed using nail polish and left to dry for around 15 minutes. Slides were covered with aluminum foil during this time to prevent photobleaching. After nail polish completely dried, slides were stored at -20°C. Cells were visualized using the confocal microscope in Bilkent University, UNAM.

2.3 Gene Set Enrichment Assay (GSEA)

From our previously performed RNA-seq analysis which was performed within the scope of another TUBITAK 1001 project (119Z405), we had wild type versus setd3Δ log fold change (LogFC) values of each transcript at the mESC state, as well as at the 2nd, 3rd and 4th days of endoderm differentiation. A ranked file was prepared for each day, listing LogFC values from highest to lowest. Positive LogFC values indicate higher expression levels in wild type cells when compared to setd3Δ cells, negative LogFC values indicate higher expression levels in setd3Δ cells compared to wild type cells. GSEA analysis was performed using GSEA 4.3.2 software, "GSEAPreranked" tool. All conditions were used as recommended and data set was used "as is" in the original format ("No_collapse").

2.4 Proximity Ligation Assay (PLA)

Before starting the experiment, 12 mm round coverslips (Cat. No.: 126.02.024, Isolab) were placed into 12-well TC plates and gelatinized using 0.1% gelatin solution. 1.5 x 10^5 live wild type and 1.5 x 10^5 live setd3Δ mESCs grown in standard serum-containing mESC medium and 1.5 x 10^5 live wild type mESCs grown in 2i4 medium were seeded onto coverslips. Wild type mESCs grown in standard serum-containing mESC medium were seeded to 2 wells. One coverslip was used to observe SETD3-β-catenin interaction and other was used as a no primary antibody negative control. After seeding the cells onto coverslips media was completed to 1 mL by using the growth medium. Cells were incubated in a 37°C, 5% CO_2 TC incubator for around 24 hours. After 24 hours, coverslips containing the cells were transferred into 10 cm petri dishes and a humidity chamber was made using wet paper towels to avoid drying of cells. To remove remaining media, cells were briefly washed with 250 µL 1X PBS. PBS was removed and 250 µl 4% PFA was used to fix the cells and incubated for 15 minutes at RT. Later, PFA was removed by filter paper and 250 µl 0.1% TritonX-100 was incubated at RT for 5 minutes twice, to permeabilize the cells. 0.1% TritonX-100 was replaced with 40 µl Duolink Blocking Solution which was provided by the Duolink PLA Kit (Cat. No.: DUO92008, Sigma) and incubated in 37°C, 5% CO_2 TC incubator for 30 minutes. After completely removing blocking solution 40 µl primary antibody solution which contains SETD3, and β-catenin antibodies diluted in antibody diluent, provided by the Duolink In Situ PLA Probe Anti-Mouse PLUS Kit (Cat. No: DUO92001-30RXN, Sigma) was added on top of cells. For negative control, 40 µl Duolink Blocking solution (without primary antibody) was added on top of cells. Information about antibodies used for PLA experiment can be found in Appendices C. Cells were incubated overnight at 4°C inside the humidity chamber. Next day, primary antibody solution was removed by filter paper and cells were washed three times for 5 minutes at RT with 100 µl Wash Buffer A which was provided by Duolink In Situ Red Starter Kit Mouse/Rabbit (Cat. No.: DUO92101- 1KT, Sigma). Duolink In Situ PLA Probe Anti-Mouse PLUS (Cat

No.: DUO92001-30RXN, Sigma) which was used for β-catenin antibody, and Duolink In Situ PLA Probe Anti-Rabbit MINUS (Cat. No.: DUO92005-30RXN, Sigma) which was used for Setd3 antibody were diluted with antibody diluent provided by the Duolink PLA Kit. 40 µL plus-minus probe solution was put onto coverslips and incubated at 37°C, 5% CO_2 TC incubator in humidity chamber for 1 hour. Later, cells were washed again for three times, 5 minutes with 250 µl Wash Buffer A and 40 µl ligation buffer together with ligase which were provided by the Duolink PLA Kit was added onto coverslips. Humidity chamber was incubated in 37°C, 5% CO_2 TC incubator for 30 minutes. After performing washing steps with Wash Buffer A, 40 µL amplification buffer together with polymerase, provided by the kit, were added onto coverslips, humidity chambers were covered with aluminum foil and incubated in 37°C, 5% CO_2 TC incubator for 2 hours. Coverslips were washed with 250 µL Wash Buffer B, provided by Duolink In Situ Red Starter Kit Mouse/Rabbit, twice for 10 minutes. Wash Buffer B was diluted to 0.01x with 1X PBS and coverslips were washed with it for 1 minute. Finally, coverslips were dried completely with filter paper and 40 µl DAPI containing mounting medium was added on top of cells for mounting. Coverslips were placed onto slides as cells were facing down. The edges of the coverslips were sealed using nail polish and left to dry for around 15 minutes. Slides were covered with aluminum foil during this time to prevent photobleaching. After nail polish completely dried, slides were stored at -20°C. Cells were visualized using the confocal microscope in Bilkent University, UNAM. Figure enhancements were done in Image J software and each enhancement was performed at the same level.

2.5 Immunocytochemistry (ICC) Assay

Before starting the experiment, 12 mm round coverslips (Cat. No.: 126.02.024, Isolab) were placed into 12-well TC plates and gelatinized using 0.1% gelatin solution. $1.5x10^5$ live wild type and setd3Δ mESCs grown in standard serum-containing mESC medium and $1.5x10^5$ live wild type and setd3Δ mESCs grown in

2i4 medium were seeded onto coverslips inside the 12-well TC plates. setd3Δ mESCs grown in 2i4 medium were seeded onto 2 coverslips. One coverslip was used as a no primary antibody negative control. Media was completed to 1 mL by using the medium that cells were grown in. Cells were incubated in a 37°C, 5% CO_2 TC incubator for around 24 hours. After 24 hours, coverslips containing the cells were transferred into 10 cm petri dishes and a humidity chamber was made using wet paper towels to avoid drying of cells. To remove remaining media coverslips were briefly washed with 1X PBS. To fix the cells, 250 µl 4% PFA was used and incubated for 15 minutes at RT. Later, PFA was removed by filter paper and 250 µl 0.1% TritonX-100 was incubated for 5 minutes twice at RT, to permeabilize the cells. Then, it was removed, 40 µl 10% BSA solution (*see* Appendices B) was added and incubated in 37°C, 5% CO_2 TC incubator for 30 minutes. Later, blocking solution was discarded and 40 µl primary antibody diluted with 1% BSA solution was added on top of cells. Information about antibodies that were used in ICC experiments can be found in Appendices C. For negative control, 40 µl 1% BSA solution (without primary antibody) was added on top of setd3Δ cells grown in 2i4 medium. Cells were incubated overnight at 4°C inside the humidity chamber. Next day, primary antibody solution was removed by filter paper and cells were washed three times, for 5 minutes at RT with 250 µl 1X PBS. Then, secondary antibodies were diluted with 1% BSA solution and coverslips were incubated with 40 µL secondary antibody solution for 1 hour in the 37°C, 5% CO_2 TC incubator. Petri dishes were covered with aluminum foil before placing into incubator to prevent photobleaching. After incubation cells were washed with 250 µl 1X PBS for 10 minutes, three times at RT. As final washing step was over, excess PBS was removed and 40 µl DAPI containing mounting medium was added on top of cells for mounting. Coverslips were placed onto slides as cells were facing down. The edges of the coverslips were sealed using nail polish and left to dry for around 15 minutes. Slides were covered with aluminum foil during this time. After nail polish completely dried, slides were stored at -20°C. Cells were visualized using the confocal microscope in Bilkent University, UNAM.

Figure enhancements were done in Image J software and each enhancement was performed at the same level.

2.6 Expression Studies

2.6.1 Protein Studies

2.6.1.1 Whole Cell Protein Sample Preparation

Wild type and setd3Δ mESCs grown in 2i4 or standard serum-containing mESC medium were collected into an eppendorf and washed with ice cold 1X PBS, three times. After last centrifuge, which was performed at 300 g, 4°C for 5 minutes, PBS was completely aspirated. Pellets were snap frozen in liquid nitrogen and stored at -80°C. Before loading to SDS-PAGE, cells were thawed on ice and resuspended in 2x Laemmli Sample Buffer solution (*see* Appendices B). Then, boiled at 95°C for 5 minutes, incubated on ice for 2 minutes and centrifuged at 10 000 rpm for 1 minute.

2.6.1.2 Nuclear and Cytoplasmic Fraction Preparation

Cells were pelleted as described in whole cell protein sample preparation section and stored at -80°C. Nuclear and cytoplasmic fractions were prepared using Universal Magnetic Co-IP Kit (Cat. No.: 54002, Active Motif) following the suggested protocol of the kit. 5.0 x 10^6 cells were used for fractionation. Protein concentrations were measured using Pierce™ BCA Protein Assay (Bicinchoninic Acid Assay) (Cat. No.: 23227, Thermo Fischer). Before loading to gel required amount of protein was taken and mixed with 4x Laemmli Sample Buffer solution (*see* Appendices B). Then, samples were boiled at 95°C for 5 minutes, incubated on ice for 2 minutes and centrifuged at 10 000 rpm for 1 minute.

2.6.1.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

1.5 x 10^5 live cells from whole cell lysates in 2x Laemmli Sample Buffer solution (*see* Whole Cell Protein Sample Preparation) and 8 µg protein from nuclear and cytoplasmic fractions in 4x Laemmli Sample Buffer solution (*see* Nuclear and Cytoplasmic Fraction Preparation) were loaded to 12% polyacrylamide gel and run at 100V for around 2 hours. Proteins on the gel were transferred to nitrocellulose membrane (Cat. No.: GE10600004, Sigma) by using Bio-Rad TransBlot Turbo Transfer System (Cat. No.: 1704150, Bio-Rad) and alternative transfer buffer (*see* Appendices B) with semi-dry transfer method. To block the membrane, 5% skim milk solution (*see* Appendices B) was incubated for 1 hour at RT. Membranes were incubated with primary antibodies for 1h at RT. After incubation, membranes were washed for 10 minutes, three times at RT with 1X TBS-T (*see* Appendices B) and incubated for 1h at RT with suitable secondary antibodies that are conjugated with horseradish peroxidase (HRP). Used primary and secondary antibodies together with dilutions are indicated in Appendices C. Then, membranes were washed three times for 10 minutes at RT with 1X TBS-T and visualized using either Clarity Western ECL Substrate (Cat. No.: 1705060, Bio-Rad), SuperSignal^{TM} West Dura Extended Duration Substrate (Cat No.: 34075, Thermo Fischer) or Clarity Max Western ECL Substrate (Cat. No.: 1705062, Biorad) using the Biorad ChemiDoc MP Imaging System (Cat. No.: 170-8280, Biorad). Obtained data was organized using Image Lab software.

2.6.2 Gene Expression Studies

2.6.2.1 Expansion and Isolation of Plasmids

pGL3 BAR and pGL4.73 hRluc/SV40 plasmids that were obtained from the laboratory of Associate Professor Güneş Özhan (Izmir Biomedicine and Genome

Center, Dokuz Eylul University Health Campus) were transformed to DH5α bacteria. Bacteria were grown in 100 µg/mL ampicillin containing LB medium and seeded to 100 µg/mL ampicillin containing LB agar plates. Colonies were selected and again grown in 100 µg/mL ampicillin containing LB medium. Plasmids were isolated using PureLink™ HiPure Plasmid Filter Midiprep Kit (Cat. No.: K210014, Invitrogen) using the manufacturers protocol. Concentration of plasmids were measured using NanoDrop.

2.6.2.2 Transfection of mESCs

Wild type and setd3Δ ESCs were grown in either 2i4 or standard serum-containing mESC medium. As they reached 80-90% confluency they were detached from the plate using the required reagent (*see* Cell Culture). Counted using trypan blue (Cat. No.: 15250061, Gibco) and 6.9 x 10^4 live cells were seeded to a well of gelatinized 24-well TC plates. Volume of each well was completed to 500 µL using the medium that cells were grown in. 2.5 ng pGL4.73 hRluc/SV40 (Cat. No.: E6911, Promega) and 47.5 ng pGL3 BAR plasmid (Vincan, 2008) per well were co-transfected with Lipofectamine 3000 (Cat. No: L30000008, Thermo Fischer Scientific) reagent following the manufacturers protocol.

2.6.2.3 Dual Luciferase Assay

24 hours after transfection, cells were washed with 1X PBS and 100 µL Passive Lysis Buffer provided by the Dual-Luciferase® Reporter Assay System Kit (Cat. No.: E1910, Promega) was incubated with cells on a shaker at RT for 20 mins. Lysates were collected and stored at -80°C. Readings were taken by using 25 µL cell lysate and 25 µL from each reagent, LarII and Stop&Glo which were provided by the kit. SpectraMax ID3 machine (Cat. No.: 735-0391, VWR) was used to take the measurements. Results were organized and statistical analysis (t-test) was performed using GraphPad Prism software.

CHAPTER 3

RESULTS

3.1 Phalloidin Staining

In our laboratory we are investigating the role of SETD3 on mESC differentiation. SETD3 is both localized in the nucleus and in the cytoplasm. As a histone methyltransferase it can dimethylate H3K4 and H3K36 in the nucleus (Eom et al., 2011; Kim et al., 2011). Also, it interacts with TFs such as FoxM1 and regulate gene expression (Cohn et al., 2016; Jiang et al., 2018). Recently its cytoplasmic function was identified as an actin specific histidine N-methyltransferase (Kwiatkowski et al., 2018; Wilkinson et al., 2019).

Actin is a monomeric globular protein (G-actin) which, under physiological conditions, binds to ATP and polymerizes into stable actin filaments (F-actin). Actin polymerization dynamics are tightly regulated (Kwiatkowski et al., 2018). Methylation of the conserved His73 residue of actin was found to be stabilizing F-actin, as increased depolymerization was observed in the absence of its methylation (Nyman et al., 2002). Therefore, in the same research, Kwiatkowski et al. performed phalloidin staining to observe F-actin levels and reported that Setd3 deficient HAP1 cells had less F-actin compared to wild type cells.

Our previous findings showed that absence of SETD3 protein causes a defect in endoderm differentiation of mESCs, suggesting a role of Setd3 in endoderm differentiation. According to our preliminary data, we hypothesized that endoderm differentiation defect observed in setd3Δ mESCs is result altered levels and/or localization of β-catenin, resulting in insufficient activation of the canonical Wnt signaling pathway in the absence of SETD3.

Before investigating this hypothesis, we wanted to observe the actin cytoskeleton in wild type, setd3Δ and setd3Δ + pEF1α-Setd3 (rescue) mESCs, to observe whether this defect might stem from the cytoplasmic function of SETD3 as an actin methyltransferase. Therefore, to observe the actin cytoskeleton we performed phalloidin staining with wild type, setd3Δ and rescue mESCs grown in standard serum-containing mESC medium, as differentiation defect was observed in setd3Δ mESCs grown in this medium. Rescue mESCs were produced in the scope of our previous TUBITAK 1001 project and western blot experiments showed that expression levels of SETD3 was similar to wild type cells, both in the nucleus and in the cytoplasm (Data not shown). As SETD3 is found to be an actin methyltransferase and methylation of actin cytoskeleton affects the polymerization of actin fibers, if the cytoplasmic function was the cause, we would expect to see a decrease in actin cytoskeleton in setd3Δ mESCs and similar levels of actin cytoskeleton in wild type and rescue mESCs. Results showed that there is no significant difference in the F-actin levels between these three cell lines (*see* Figure 3.1), suggesting that the defect observed in setd3Δ mESCs are possibly not due to the cytoplasmic function of SETD3 and more likely to be due to its nuclear function. However, future experiments are required to validate this.

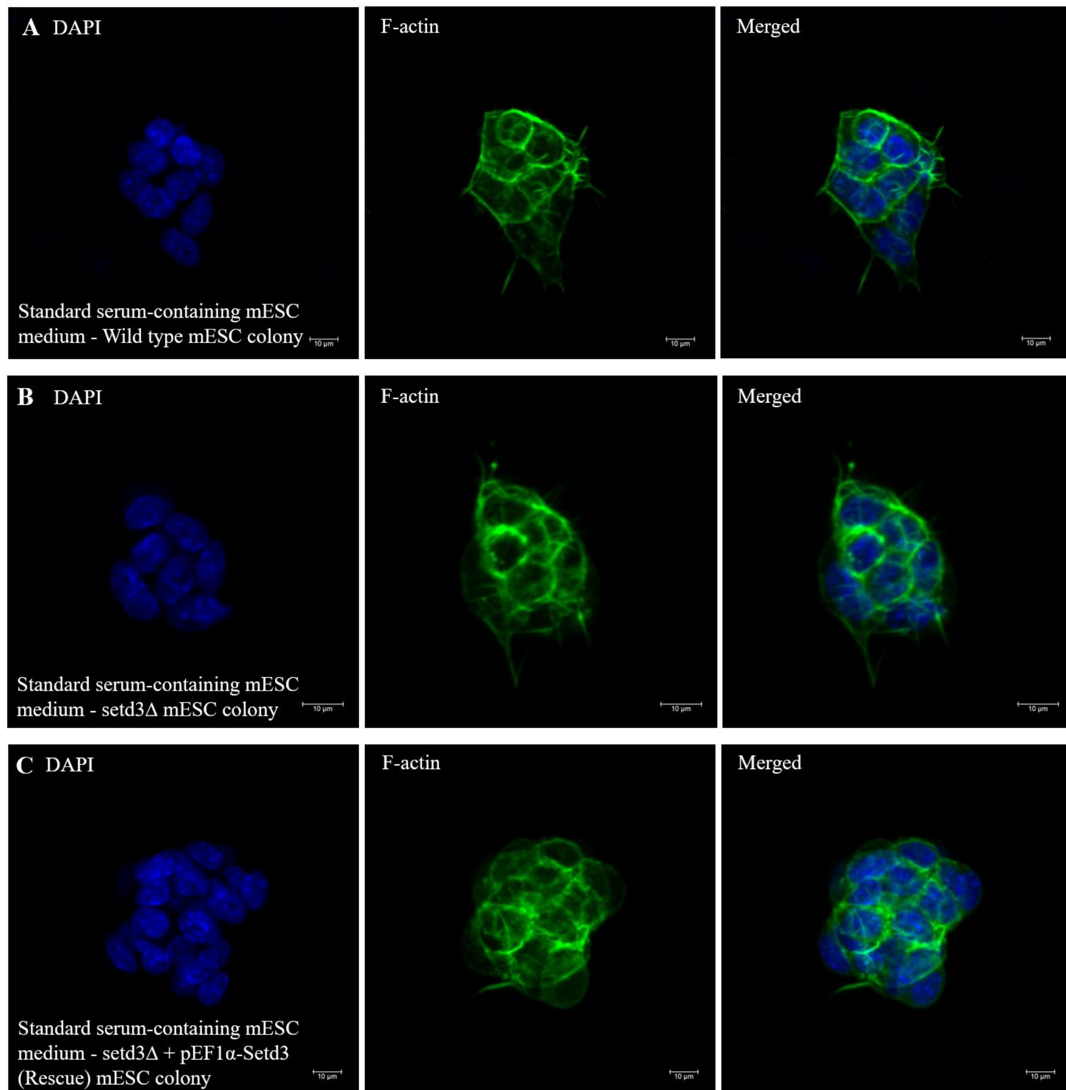

Figure 3.1. Actin cytoskeleton is not affected from the absence of SETD3. First column shows DAPI stained nuclei (blue) in the mESC colony. Second column shows the F-actin stain (green), and third column shows the merged image. Actin cytoskeleton in **A.** Wild type, **B.** setd3Δ, **C.** setd3Δ + pEF1α-Setd3 (Rescue) mESC colonies grown in standard serum-containing mESC medium. Scale bars, 10μm.

3.2 Gene Set Enrichment Analysis (GSEA)

Our preliminary data showed that presence of SETD3 protein has an important role in endoderm differentiation (*see* Preliminary Data, Figure 1.1). To understand its effect further, within the scope of our TUBITAK 1001 project (119Z405), RNA samples were collected from mESC state and from 2nd, 3rd and 4th, days of endoderm differentiation of wild type and setd3Δ cells to generate an RNA-seq library. In each day of differentiation, differentially expressed genes (DEGs) between wild type and setd3Δ mESCs were identified and the signaling pathways that they are enriching were found using the KEGG database. According to this analysis, Wnt signaling pathway was found to be enriched by genes that are expressed more in wild type cells when compared to setd3Δ cells (upregulated) at the mESC state and at the 2nd and 3rd days of endoderm differentiation (*see* Preliminary Data, Figure 1.2) This result suggested that higher expression of Wnt signaling pathway related genes observed in wild type cells are important for proper differentiation towards endoderm lineage and differentiation defect observed in setd3Δ cells might be due to inability of these cells to express these genes in the required level or at the required time. To validate the enrichment results observed with KEGG analysis, Gene Set Enrichment Analysis (GSEA) was performed by using Pre-ranked GSEA tool. Wild type versus setd3Δ LogFC values were ranked from highest to lowest, higher values indicating a higher expression in wild type cells while lower values indicating higher expression in setd3Δ cells. Consistent with KEGG analysis results, Wnt signaling pathway was observed to be significantly enriched by upregulated genes at the 2nd and 3rd day of endoderm differentiation, even though not at the mESC state (*see* Figure 3.2). Enrichment scores and normalized p-values are indicated in Table 2. As GSEA collects data from many databases which have different number of pathway attributed genes, same pathway from different databases can appear in the enrichment analysis results as happened in this case. Both KEGG and GSEA results indicate that presence of SETD3 might have a role in the activity of Wnt signaling pathway during endoderm differentiation.

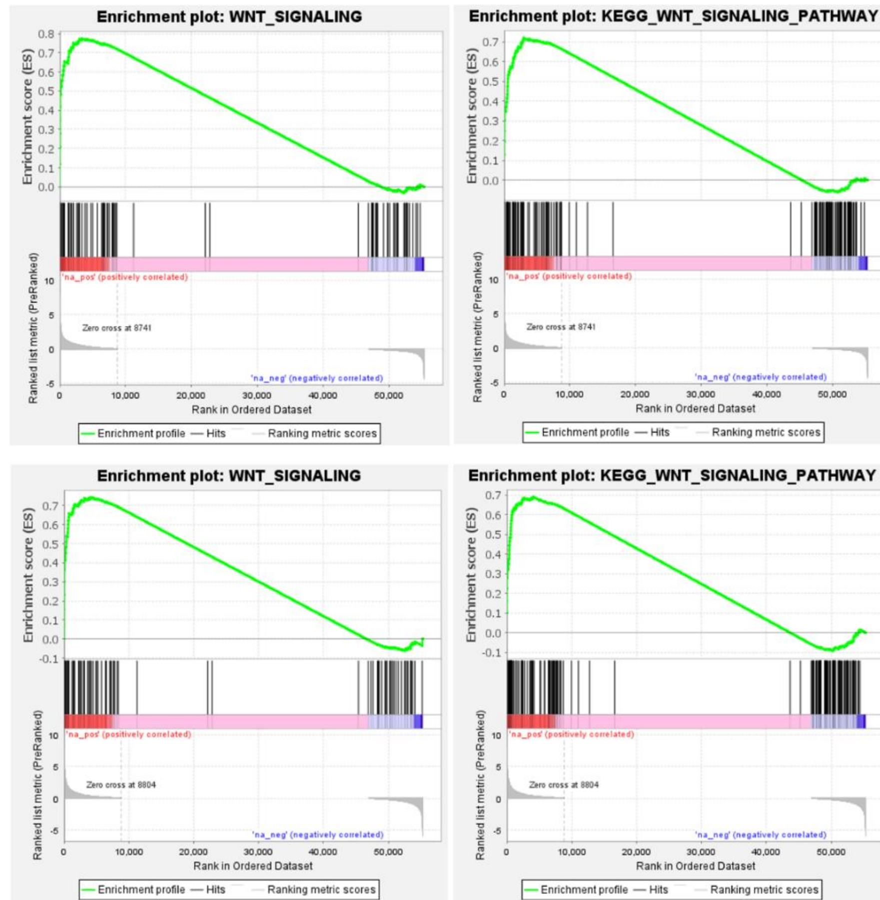

Figure 3.2. Pre-ranked GSEA performed using expression data from RNA-seq analysis showed that Wnt signaling pathway is enriched by genes that are upregulated in wild type cells at the 2nd and 3rd days of endoderm differentiation. Top panels indicate enrichment at 2nd day, and bottom panel indicate enrichment at 3rd day of endoderm differentiation.

Table 2. Pre-ranked GSEA results showed significant enrichment of the Wnt signaling pathway.

Differentiation Day	***Geneset***	***Enrichment Score***	***Normalized p-value***
2nd day	Wnt_Signaling	0.77	0.011
2nd day	KEGG_Wnt_Signaling _Pathway	0.72	0.015
3rd day	KEGG_Wnt_Signaling _Pathway	0.69	0.013
3rd day	Wnt_Signaling _Pathway	0.74	0.022

3.3 Proximity Ligation Assay (PLA)

Effect of SETD3 on endoderm differentiation is hypothesized to be due to its effect on the canonical Wnt signaling pathway. Even though as a result of our previously performed IP-MS analysis SETD3 was found to be interacting with proteins that are involved in canonical Wnt signaling pathway (*see* Preliminary Data, Table 1), interaction of SETD3 and β-catenin was not detected. Therefore, proximity ligation assay (PLA) was performed to investigate the physical interaction of SETD3 with the key transcription factor of the canonical Wnt signaling pathway, β-catenin.

PLA is a sensitive technique used to observe the physical interaction of proteins within the cell that are at a maximum distance of 40 nanometers from each other. In this technique, used secondary antibodies can recognize the primary antibodies of target proteins and they are covalently linked to different DNA oligos which are complementary to each other. As two target proteins are in close proximity, circular DNA synthesis takes place. Synthesized DNA is observed as dots inside the cell. Circular DNA synthesis can demonstrate rare or transient protein interactions better, with the help of amplification of the signal that arise from interaction of two proteins (Fredriksson et al., 2002; Greenwood et al., 2015; Söderberg et al., 2006; Weibrecht et al., 2010).

In PLA experiment, wild type mESCs grown in standard serum-containing mESC medium were used as a no primary antibody control to observe nonspecific signal coming from secondary antibodies (*see* Figure 3.3A). setd3Δ mESCs grown in standard serum-containing mESC medium were used as a negative control as interaction of SETD3 and β-catenin was investigated, and cell line did not have SETD3 protein (*see* Figure 3.3B). No signal was detected in either of the negative controls, indicating that obtained signal is directly coming from the interaction of SETD3 and β-catenin. Result of PLA experiment, performed with wild type mESCs grown in either standard serum-containing mESC medium or 2i4 medium showed that SETD3 interacts with β-catenin in wild type mESCs (*see* Figure 3.3C-D). As GSK3β is a component of the “destruction complex” which phosphorylates and leads

to degradation of β-catenin, when 2i4 medium that contains GSK inhibitor is used, β-catenin is no longer degraded. Consistent with this, more SETD3 - β-catenin interaction is observed in wild type mESCs grown in 2i4 medium (*see* Figure 3.3D).

The role of SETD3 in mESC differentiation might be both related to canonical and non-canonical Wnt signaling pathways. However, additional to our preliminary data, the observation that SETD3 and the key transcription factor of the canonical Wnt signaling pathway, β-catenin, is interacting, more strongly support the relationship between SETD3 and the canonical Wnt signaling pathway. Therefore, we prioritized understanding the possible involvement of SETD3 on the canonical Wnt signaling pathway in our studies as we have already hypothesized.

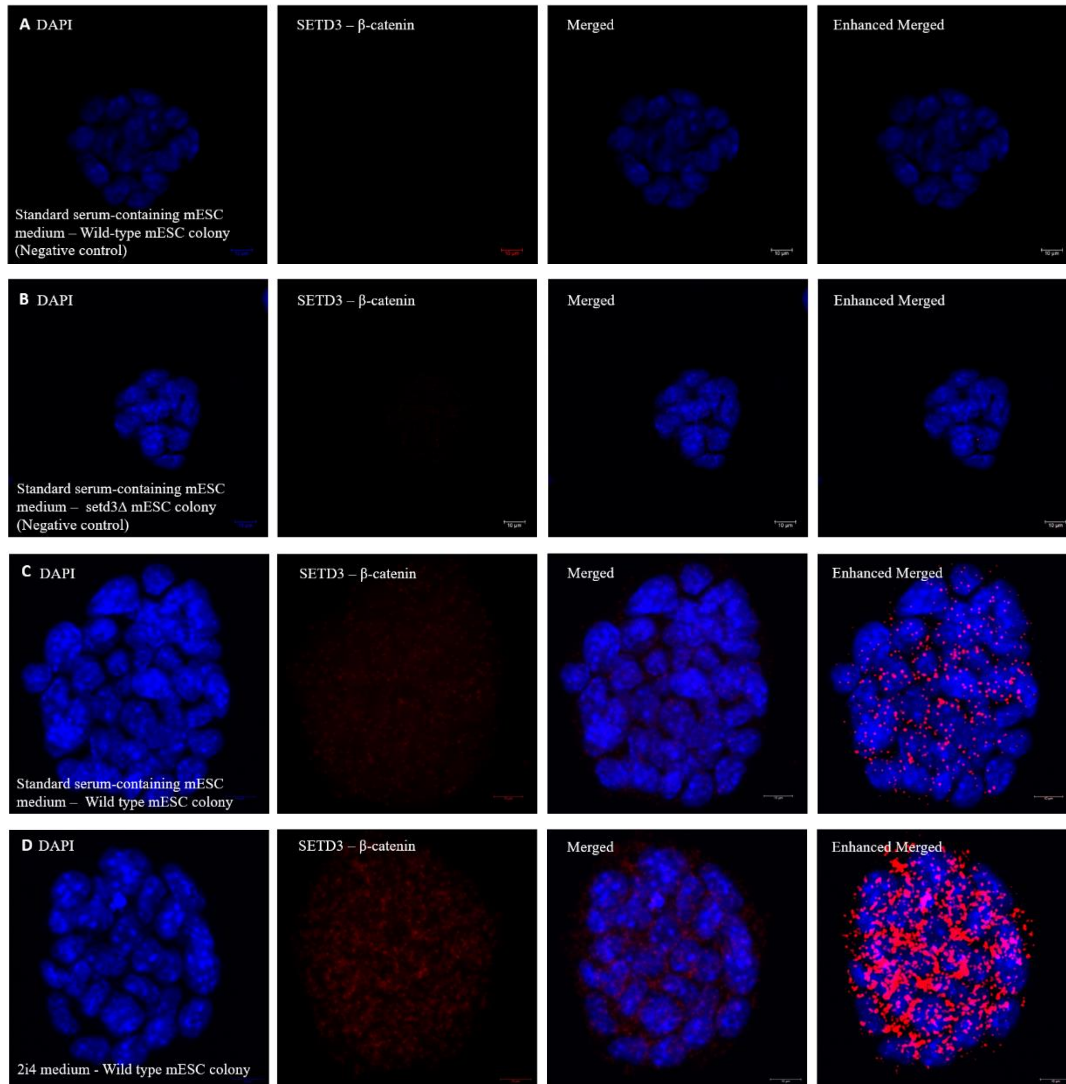

Figure 3.3. Proximity Ligation Assay (PLA) showed that SETD3 and β-catenin interacts in mESCs. First column shows DAPI stained (blue) nuclei of the cells inside the mESC colony. Red dots in the second column shows the interaction of SETD3 and β-catenin proteins. Third column shows the merged image, and fourth column shows the merged image with enhanced red signal. **A**. Wild type mESCs grown in standard serum containing mESC medium, used as no primary antibody negative control (only secondary antibodies were used). **B.** setd3Δ mESCs grown in standard serum-containing mESC medium, used as negative control. SETD3 - β-catenin interaction in wild type mESCs **C.** grown in standard serum containing mESC medium, **D.** grown in 2i4 medium. Scale bars, 10μm. First replicate First replicate of PLA experiments performed with wild type cells either grown in standard serum-containing mESC medium or 2i4 medium were performed by my lab mate Dersu Sezginmert. Enhanced images were prepared with Image J software using same parameters for every mESC colony.

3.4 Determining the Cellular Level and Localization of β-catenin in Wild type and setd3Δ mESCs Grown in Either Standard Serum-Containing mESC Medium or 2i4 Medium

3.4.1 Determining Cellular Level and Localization of β-catenin in mESCs Using Immunocytochemistry (ICC) Assay

Interaction of SETD3 and β-catenin suggests a possible role of the complex which might be resulting in the differentiation defect observed when SETD3 is absent. Therefore, we first decided to determine the effect of SETD3, on β-catenin level and localization, using mESCs either grown in standard serum-containing mESC medium or 2i4 medium by performing ICC assay.

As a no primary antibody negative control, setd3Δ mESCs grown in 2i4 medium were used and β-catenin signal was not detected (*see* Figure 3.4A). This indicates that the obtained signal specifically comes from the β-catenin antibody. ICC assay results showed that β-catenin levels in mESCs grown in 2i4 medium were higher than mESCs grown in standard serum-containing mESC medium regardless of their SETD3 status (see Figure 3.3). This was expected due to β-catenin stabilizing effect of GSK3 inhibitor found in 2i4 medium. As setd3Δ mESCs grown in 2i4 medium did not show a defect in differentiation, it was anticipated that β-catenin levels in mESCs grown in this medium would be unchanged. As predicted, β-catenin levels in both wild type and setd3Δ mESCs grown in this medium were observed to be similar (*see* Figure 3.3B and C). Differentiation defect detected in setd3Δ mESCs grown in standard serum-containing mESC medium was hypothesized to be the result of low levels of β-catenin in these cells. According to this, lower levels of β-catenin were expected in setd3Δ mESCs grown in standard serum-containing mESC medium when compared to other three groups. However, mESCs grown in standard serum-containing mESC medium showed similar levels of β-catenin regardless of their SETD3 status (*see* Figure 3.3D and E). Even though an overall nuclear, cytoplasmic and plasma membrane localization of β-catenin was detected in all

mESCs, nuclear localization could not be clearly distinguished from the other signals even in mESCs grown in 2i4 medium. As signal is not strictly coming from the nucleus, and signal from the cytoplasm and plasma membrane is high, relatively lower nuclear β-catenin signal could not be directly detected when β-catenin signal is merged with DAPI staining. Additionally, the 3D structure of the ESC colonies made it harder to interpret the localization of the signal. Therefore, we decided to investigate this further by performing Western blot experiments with whole cell lysates as well as nuclear and cytoplasmic fractions of the mESCs.

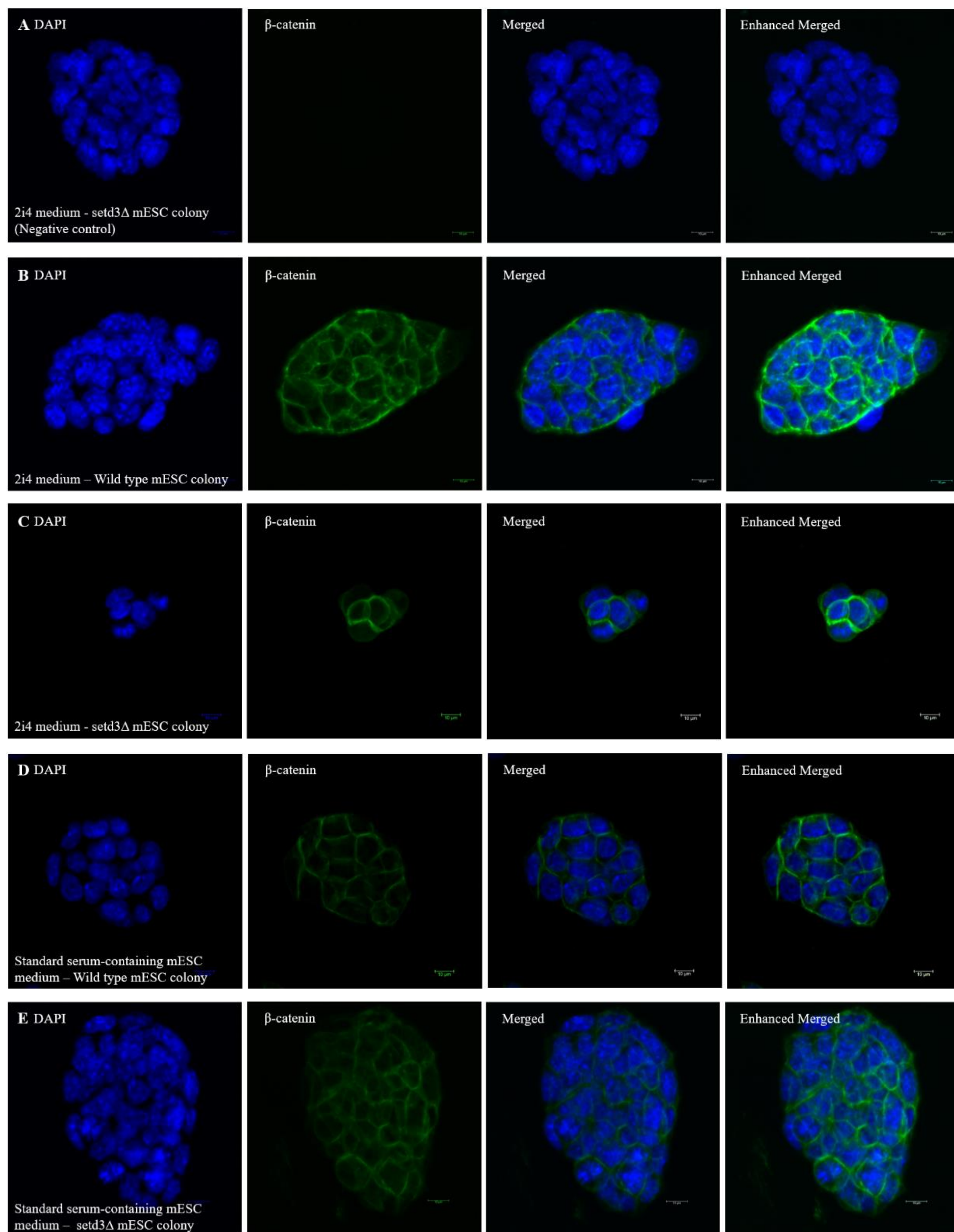

Figure 3.4. Cellular levels of β-catenin are observed to be higher in mESCs grown in 2i4 medium regardless of their SETD3 status, while mESCs grown in same medium did not show any significant differences in β-catenin levels in immunocytochemistry assay. Cellular level and localization of β-catenin determined ICC in wild type or setd3Δ ESC colonies grown in standard serum-containing mESC medium or 2i4 medium. First column shows DAPI stained nuclei inside the mESC colony (blue), second column shows β-catenin protein signal (green), third column shows the merged image, and fourth column shows the merged

image with enhanced β-catenin signal. β-catenin levels in **A.** No primary antibody negative control, **B.** Wild type mESC colony grown in 2i4 medium, **C.** setd3Δ mESC colony grown in 2i4 medium, **D.** Wild type mESC colony grown in standard serum-containing mESC medium, **E.** setd3Δ mESC colony grown in standard serum-containing mESC medium. Figure is representative of two replicates. Scale bars, 10 μm. Enhanced images were prepared with Image J software using same parameters for every mESC colony.

3.4.2 Determining Cellular Level and Localization of β-catenin in mESCs Using Western Blot Technique

ICC assay showed higher β-catenin levels in mESCs grown in 2i4 medium compared to mESCs grown in standard serum-containing mESC medium. However, differences between wild type and setd3Δ mESCs grown in the same medium could not be observed possibly due to different localization of β-catenin. To better understand the overall differences of β-catenin levels in wild type and setd3Δ mESCs grown in either standard serum-containing mESC medium or 2i4 medium, Western blot technique was performed using whole cell lysates.

GAPDH was used as a loading control for western blot experiments performed with whole cell lysates. Consistent with ICC results (*see* Figure 3.4), Western blot experiment performed with whole cell lysates (*see* Figure 3.5A) showed that mESCs grown in 2i4 medium, regardless of their SETD3 status, showed higher β-catenin levels compared to mESCs grown in standard serum-containing mESC medium. Similar to ICC results, mESCs grown in 2i4 medium did not show any difference in β-catenin levels regardless of their SETD3 status. This result might explain the ability of setd3Δ mESCs grown in 2i4 medium to successfully differentiate into endoderm layer. If differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium is due to a change in β-catenin levels resulting from the absence of SETD3, as in our hypothesis, we would expect to observe lower levels of β-catenin in those mESCs compared to other three groups. However, both wild type and setd3Δ mESCs grown in standard serum-containing mESC medium showed similar levels of β-catenin. This suggests that differentiation defect observed in setd3Δ mESCs that are grown in standard serum-containing

mESC medium might be due to localization differences of β-catenin, instead of a change in the total β-catenin levels.

We could not clearly observe the nuclear β-catenin pool using ICC, possibly due to low level of β-catenin pool in the nucleus and higher signal coming from cytoplasm and plasma membrane. To understand the localization differences better, we further generated cytoplasmic and nuclear fractions from wild type or setd3Δ mESCs that were grown either in standard serum containing mESC medium or 2i4 medium. β-catenin levels in these fractions were investigated using Western blotting technique.

In Western blot experiments performed with cytoplasmic fractions, GAPDH was used as a loading control, and BRD2 was used to validate complete separation of cytoplasmic fraction from the nuclear fraction. As no BRD2 signal was detected, observed β-catenin bands are solely coming from the cytoplasmic β-catenin pool. Due to stabilization of β-catenin in 2i4 medium, it was expected to observe higher cytoplasmic β-catenin levels in mESCs cultured in this medium, compared to ones grown in standard serum-containing mESC medium. As expected, both mESCs grown in 2i4 medium, regardless of their SETD3 status, showed higher levels of β-catenin (*see* Figure 3.4B). No consistent differences in cytoplasmic β-catenin level were observed between wild type and setd3Δ mESCs grown in the same media, either in 2i4 medium or standard serum-containing mESC medium. As GSK3 leads to nuclear localization of β-catenin, we expected to see differences in nuclear β-catenin levels, therefore, observing similar levels of cytoplasmic β-catenin grown in same media is not surprising.

In Western blot experiments performed with nuclear fractions, HDAC1 was used as a loading control and GAPDH was used to validate complete separation of nuclear fraction from the cytoplasmic fraction. As no signal was detected from GAPDH, observed β-catenin bands are solely coming from the nuclear β-catenin pool. Since GSK3 inhibitor found in the 2i4 medium prevents degradation of β-catenin, it goes to the nucleus to perform its function (Valenta et al., 2012).

Accordingly, higher levels of β-catenin were expected in the nuclear fraction of mESCs grown in 2i4 medium, compared to ones grown in standard serum-containing mESC medium. Additionally, since both wild type and setd3Δ mESCs grown in 2i4 medium could successfully differentiate into endoderm layer, similar levels of nuclear β-catenin were expected. As predicted, mESCs grown in 2i4 medium showed similar levels of β-catenin and their level were higher than the levels observed in mESCs cultured in standard serum-containing mESC medium (*see* Figure 3.4C). If the differentiation deficiency observed in setd3Δ mESCs grown in standard serum-containing mESC medium is not due to a change in the total β-catenin level, but instead, due to the inability of β-catenin to localize to nucleus, we would expect to see less β-catenin in the nuclear fraction of these cells. As expected, setd3Δ mESCs grown in standard serum-containing mESC medium showed lower levels of β-catenin compared to other three groups. With KEGG analysis (*see* Preliminary Data, Figure 1.2) and GSEA results (*see* Figure 3.2), it was observed that during endoderm differentiation, expression of Wnt pathway related genes were lower in setd3Δ mESCs grown in standard serum-containing mESC medium compared to wild type cells. As nuclear β-catenin pool controls the expression of canonical Wnt signaling pathway target genes, the result of Western blot experiment performed with nuclear fractions is consistent with this observation. Overall, data from western blot experiments performed using nuclear fractions indicate that presence of SETD3 protein influences nuclear levels of β-catenin at least in mESCs grown in standard serum-containing mESC medium.

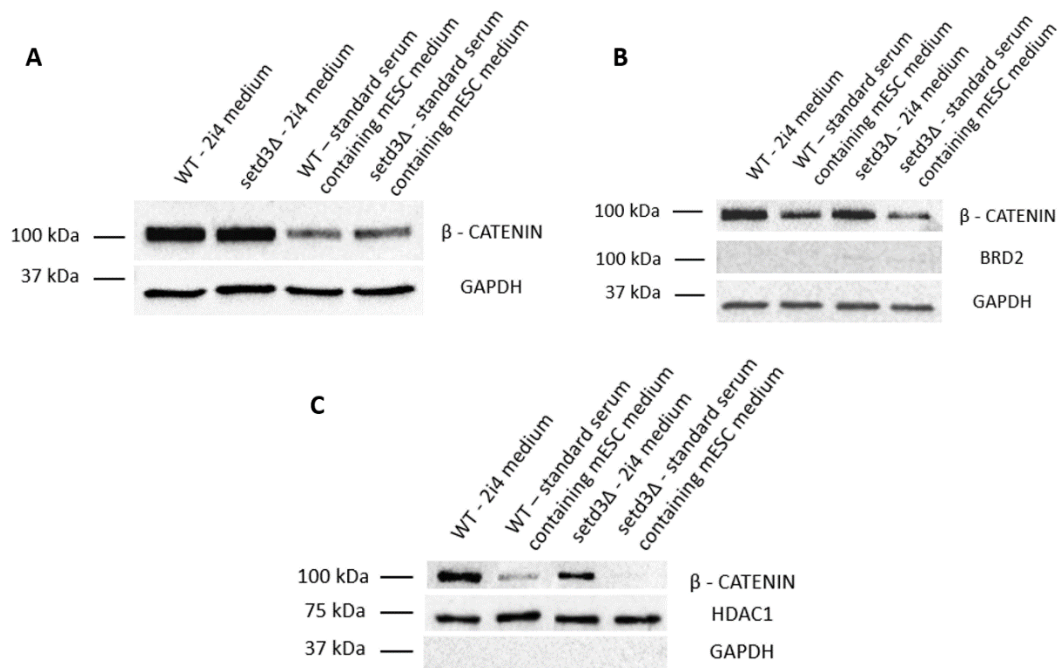

Figure 3.5. As a result of western blotting experiments, β-catenin levels in whole cell lysates and cytoplasmic fractions of wild type and setd3Δ mESCs grown in the same medium are observed to be similar, but lower nuclear β-catenin levels are detected in setd3Δ mESCs grown in standard serum-containing mESC. Levels and localization of β-catenin in wild type and setd3Δ mESCs which were either grown in standard serum-containing mESC medium or 2i4 medium were determined by western blotting experiments. **A.** β-catenin levels in whole cell lysates. GAPDH was used as a loading control. **B.** β-catenin levels in cytoplasmic fractions. GAPDH was used as a loading control. Brd2 was used to observe nuclear contamination in the cytoplasmic fraction. **C.** β-catenin levels in nuclear fractions. HDAC1 was used as a loading control. GAPDH was used to observe cytoplasmic contamination in nuclear fraction. Figure represents three independent biological replicates.

3.5 Determining the nuclear activity of β-catenin in Wild type and setd3Δ mESCs grown in either 2i4 Medium or Standard Serum-containing mESC medium

In figure 3.5C, it was observed that nuclear localization of β-catenin was lower in setd3Δ mESCs grown in standard serum-containing mESC medium, compared to other three groups. It also suggests a lower canonical Wnt signaling activity in these mESCs. To test the nuclear activity of β-catenin as a transcription factor, which is mediated by TCF/LEF transcription factors, a dual luciferase

reporter assay was performed with wild type and setd3Δ mESCs grown in either 2i4 medium or standard serum-containing mESC medium.

As an internal control, renilla reporter plasmid (pGL4.73 hRluc/SV40) which contains a SV40 promoter was cotransfected with the canonical Wnt reporter plasmid (pGL3 BAR). SV40 promoter activity is not affected from presence of SETD3 or the growth medium, therefore, expression from this plasmid is always the same. Consequently, renilla luciferase, which is controlled by that promoter, was used to detect the amount of plasmid that entered to the cells, as an internal control. Canonical Wnt reporter plasmid has 12 TCF binding motifs which contains unique five-nucleotide linkers in between. β-catenin binds to these motifs together with TCF to activate transcription of its target genes. TCF binding sites are inserted upstream of the Promega's minP minimal promoter to obtain a functional promoter which controls the expression of firefly luciferase (Vincan, 2008). As β-catenin levels in the nuclear fraction of mESCs grown in 2i4 medium was higher than ones grown in standard serum-containing mESC medium (*see* Figure3.5C), it was expected to observe higher Wnt/β-catenin signaling pathway activity in cells grown in 2i4 medium. As predicted and, consistent with the ICC assay (*see* Figure 3.4) as well as western blot results (*see* Figure 3.5), both mESCs cultured in 2i4 medium showed significantly higher canonical Wnt pathway activity when compared to their counterparts grown in standard serum-containing mESC medium (*see* Figure 3.6). These data indicate that, high level of β-catenin detected in the nuclear fraction of mESCs grown in 2i4 medium, due to the effect of GSK3 inhibitor, is increasing the activity of canonical Wnt signaling pathway. Since setd3Δ mESCs grown in standard serum-containing mESC medium showed less β-catenin in the nuclear fraction, compared to wild type mESCs grown in that medium, they predicted to have lower Wnt/β-catenin signaling activity. As expected, wild type mESCs grown in standard serum-containing mESC medium showed significantly higher Wnt/β-catenin activity than setd3Δ mESCs grown in the same medium. This result supports the findings of the western blot experiments performed using nuclear fractions, as lower levels of nuclear β-catenin suggest a lower canonical Wnt signaling activity. Since

nuclear β-catenin levels of mESCs grown in 2i4 medium were similar to each other, also similar levels of canonical Wnt pathway activity were expected. Surprisingly, setd3Δ mESCs grown in 2i4 medium showed significantly less Wnt/β-catenin signaling activity compared to wild type mESCs grown in that medium. This indicates that even though 2i4 medium rescues the endoderm differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium, and similar β-catenin levels were observed in western blotting experiments between wild type and setd3Δ mESCs, lack of Setd3 gene still affects the ability of TCF/LEF mediated canonical Wnt signaling pathway activity. Overall, these results suggest that presence of SETD3 protein has an effect on TCF/LEF mediated canonical Wnt signaling pathway activity regardless of the growth medium.

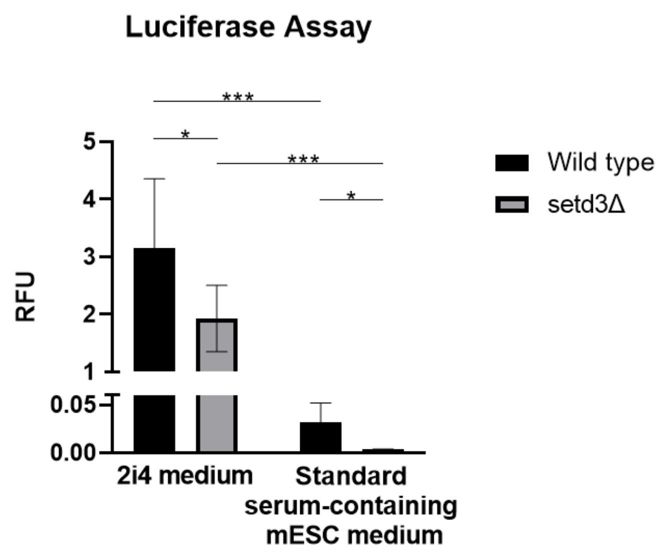

Figure 3.6. Growth medium (2i4 medium or standard serum-containing mESC medium) and presence of SETD3 affects the nuclear activity of β-catenin. Firefly luciferase activity which shows TCF/LEF mediated Wnt/β-catenin signaling pathway activity was normalized to the internal control (renilla luciferase activity). Statistical significance was determined by performing t-test using GraphPad Prism software. (*: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0001$). Error bars indicate ± SEM. Figure represents seven independent biological replicates. RFU: relative fluorescence unit

CHAPTER 4

DISCUSSION

Embryonic development is a complicated process which requires temporal and spatial (spatiotemporal) regulation of gene expression to generate a healthy and functioning organism. Epigenetic mechanisms, that control gene expression profiles, are primarily responsible from this regulation by changing the accessibility of the DNA as well as the chromatin structure (Hargreaves & Crabtree, 2011; Tang et al., 2010). SETD3 is one of the epigenetic factors which we have identified to have a role in mESC differentiation towards mesendoderm lineage, as Setd3 deleted (setd3Δ) mESCs could not successfully differentiate (*see* Preliminary Data, Figure 1.1). We hypothesized that this defect is due insufficient activation of canonical Wnt signaling pathway and decided to investigate it.

To pinpoint possible pathways that are involved in this differentiation defect, within the scope of our previous TUBITAK 1001 project (119Z405), KEGG analysis was performed. Results showed that Wnt signaling pathway is enriched by genes that are expressed more in wild type cells (upregulated genes) at the mESC state as well as at the 2nd and 3rd days of endoderm differentiation (see Preliminary Data, Figure 1.2). In this study we performed GSEA to validate this result. Consistently, enrichment by same set of genes was observed in 2nd and 3rd days of endoderm differentiation, even though not at the mESC state (*see* Figure 3.2 and Table 3.1). A possible explanation for this difference is that parameters used during the analysis are different and they use a different set of data. For example, while LogFC values of DEGs are given to KEGG database, LogFC values of all transcripts are given to the GSEA software without eliminating non-DEGs. The results of enrichment analysis suggests that setd3Δ cells are not able to upregulate Wnt signaling pathway related genes to the same extend as wild type cells and differentiation defect observed in these cells might be result of this lower expression.

Wnt signaling pathway is divided mainly into two as canonical Wnt signaling pathway and non-canonical Wnt signaling pathways (Komiya & Habas, 2008). SETD3 and the key transcriptional factor of canonical Wnt signaling pathway, β-catenin, share many interaction partners such as FoxM1 (Cohn et al., 2016; Jiang et al., 2018; N. Zhang et al., 2011). According to our previous nuclear mass spectrometry analysis, several other common interaction partners were also identified (*see* Preliminary Data, Table 1). Furthermore, β-catenin has histone methyltransferase co-activators such as MLL1/MLL2 SET1-type HMT complex and SET8 (Li et al., 2011; Sierra et al., 2006). Keeping these in mind, it is possible that SETD3, a SET domain containing protein, which introduces activatory modifications like H3K36me2 and H3K4me2 might be interacting with β-catenin by itself, or in a protein complex to activate the transcription of β-catenin target genes. In the absence of SETD3, this activation might not be sufficiently achieved which results with the observed differentiation defect in setd3Δ cells grown in standard serum-containing mESC medium. The finding that transcriptional activity of β-catenin was found to be important for differentiation further supports this prediction. Based on these, the role of canonical Wnt signaling pathway is prioritized. Mass spectrometry analysis did not reveal an interaction between SETD3 and β-catenin. However, PLA results showed that SETD3 and β-catenin interacts in mESCs, and interaction is even more in mESCs grown in 2i4 medium, consistent with β-catenin stabilization (*see* Figure 3.3). This interaction suggests a functional outcome which will be abolished when one of these proteins, SETD3 in our case, is deleted. The loss of functional interaction between SETD3 and β-catenin might be the reason for the observed defect in endoderm differentiation.

According to our preliminary data and PLA results, even though there might be possible involvement of non-canonical pathways we prioritized to understand the effect of SETD3 on the canonical Wnt signaling pathway, as non-canonical Wnt signaling pathways do not mediate their function through β-catenin. We hypothesized that SETD3 regulates mESC differentiation through controlling transcriptional activity of canonical Wnt signaling pathway, and the endoderm

differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium are due to inability of these cells to sufficiently activate the canonical Wnt signaling pathway.

From previous studies it is known that medium that mESCs are cultured in can affect their potency, the ability of them to differentiate into variety of specialized cell types (Gonzalez et al., 2016). As the only difference between two endoderm differentiation experiments (*see* Preliminary Data Figure 1.1 and Figure 1.3) is the medium cells were cultured in during mESC state, we decided to focus on the mESC state where cells are still pluripotent, to understand the effect of SETD3 during differentiation of mESCs into endoderm layer.

In the earlier studies, SETD3 was identified as a methyltransferase which has both histone and non-histone targets. In *Drosophila melanogaster* and *Mus musculus*, it has been shown to dimethylate histone 3 lysine 4 (H3K4me2) and histone 3 lysine 36 (H3K36me2) both in vivo and in vitro (Eom et al., 2011; Kim et al., 2011). Recent studies reported that SETD3 is catalyzing methylation of actin histidine 73 (His73). In the same studies, they suggested that SETD3 is only responsible from methylating actin and does not have a histone lysine methyltransferase activity (Kwiatkowski et al., 2018; Wilkinson et al., 2019). However, literature about the nuclear function of SETD3 is limited and more research is needed to enlighten its exact nuclear function.

To observe whether cytoplasmic function of SETD3 as an actin methyltransferase might have an effect on the observed differentiation defect of setd3Δ cells grown in standard serum-containing mESC, we wanted to check actin cytoskeleton levels. His73 methylation of actin increases polymerization and its stability. Therefore, to observe the potential role of cytoplasmic function as an actin histidine N-methyltransferase, we performed phalloidin staining and observed similar actin cytoskeleton levels in wild type, setd3Δ and rescue mESCs grown in standard serum-containing mESC medium (*see* Figure 3.1). This suggests that actin methyltransferase function of SETD3 is not the cause for the observed defect in

endoderm differentiation, and it might be the result of its nuclear function. However, it needs to be investigated further by performing additional experiments.

As we observed interaction between SETD3 and β-catenin, we decided to investigate the effect of SETD3 presence on β-catenin levels and localization. ICC assay results showed that mESCs grown in 2i4 medium had higher levels of β-catenin regardless of their SETD3 status, as expected due to presence of GSK3 inhibitor. However, differences between mESCs grown in the same medium could not be detected (*see* Figure 3.4). Particularly increased levels of β-catenin were observed in membrane and cytoplasm when cells were cultured in 2i4 medium. Yet, nuclear localization could not be distinguished from other signals. It is possibly due to low levels of β-catenin localized in the nucleus which could not be detected as signal coming from the cytoplasm and the plasma membrane higher. Additionally, 3D structure of the colonies make it harder to understand the localization. For this reason, total β-catenin levels are also investigated with western blotting experiments using whole cell lysates. Similar results were obtained with ICC assay (*see* Figure 3.5A). Therefore, we hypothesized that the defect is due to localization differences of β-catenin instead of a change in total levels.

To observe localization changes better, nuclear and cytoplasmic fractions were generated by using wild type and setd3Δ mESCs either grown in 2i4 medium or standard serum-containing mESC medium. Western blot experiments performed with cytoplasmic fractions (*see* Figure 3.5B) also showed similar results to ICC assay and Western blot analysis performed with whole cell lysates. Significantly, setd3Δ mESCs grown in standard serum-containing mESC medium showed lower nuclear levels of β-catenin compared to wild type mESCs grown in this medium (*see* Figure 3.5C). This further suggests that differentiation defect observed in these cells might be due to lower levels of nuclear β-catenin. Nuclear western blot experiment results indicate that presence of SETD3 protein influences nuclear levels of β-catenin, at least in mESCs grown in standard serum-containing mESC medium. As very small changes could not be detected with Western blotting technique which is a semi-quantitative method, there might be smaller changes in nuclear β-catenin levels

grown in 2i4 medium which could not be successfully detected using this technique (Mahmood & Yang, 2012).

Lower levels of nuclear β-catenin observed in setd3Δ mESCs grown in standard serum-containing mESC medium suggest lower canonical Wnt pathway activity. It is known that upon canonical Wnt signaling pathway activation, phosphorylation of β-catenin is inhibited, and it becomes stabilized. Once stabilized, it goes to nucleus to regulate transcription of its target genes (Komiya & Habas, 2008). As we hypothesized that setd3Δ mESCs grown in standard serum-containing mESC medium cannot sufficiently activate canonical Wnt signaling, and lower levels of nuclear β-catenin were detected in these cells, TCF/LEF mediated canonical Wnt pathway activity was checked by performing a dual luciferase assay. Both mESCs cultured in 2i4 medium showed significantly higher canonical Wnt pathway activity compared to their counterparts grown in standard serum-containing mESC medium. It suggests that higher levels of nuclear β-catenin observed in mESCs grown in 2i4 medium is also resulting in an increased canonical Wnt signaling pathway as expected. This higher activity can be explained by the presence of GSK3 inhibitor found in this medium which causes stabilization and nuclear transport of β-catenin. Interestingly, wild type mESCs grown in this medium showed significantly higher canonical Wnt signaling activity compared to setd3Δ mESCs grown in this medium, even though nuclear β-catenin levels were observed to be similar. This result suggests that although normal differentiation is observed in setd3Δ mESCs grown in this medium, absence of SETD3 is still affecting the TCF/LEF mediated canonical Wnt signaling activity. As expected due to lower nuclear β-catenin levels, setd3Δ mESCs grown in standard serum-containing mESC medium also showed significantly lower TCF/LEF mediated canonical Wnt pathway activity compared to wild type mESCs grown in this medium. This further supports our nuclear Western blot findings and hypothesis that absence of SETD3 in these cells are affecting the canonical Wnt pathway activity and the differentiation defect observed in these cells are possibly due to this effect. These results suggest that SETD3 protein has an effect

on canonical Wnt signaling pathway activity which is mediated by TCF/LEF, regardless of the growth medium.

We observed experimental variability in luciferase activity among replicates. One of the sources of variability might stem from the duration between the last medium change and the transfection day. As a result of each medium change, the activity of transcription factors changes drastically which might result in variability between replicates. Another contribution might come from colony sizes and the confluency of the cells, since intercellular interactions alter the activity of signaling pathways. Additionally, mESCs grown in the standard serum-containing mESC medium are cultured on mitomycin-C treated MEFs and right before the luciferase reporter plasmid transfection, cells are separated from mitoMEFs. However, if complete removal cannot be achieved, signal from remaining mitoMEFs might also add to the variability between experimental replicates.

The observed difference in canonical Wnt signaling pathway activity might also be due to different amount of TCF3 found in these cells. However, results of RNA-seq performed with mESCs grown in standard serum-containing mESC medium showed that none of the TCF proteins are differentially expressed between wild type and setd3Δ mESCs. Additionally, RT-qPCR of wild type and setd3Δ mESCs grown in 2i4 medium showed that there is no significant difference in TCF3 levels. Therefore, decrease in TCF proteins is not the reason of observed lower canonical Wnt signaling pathway activity in the absence of SETD3. It is also important to note that endogenous TCF/LEF activity is not faithfully reflected with the activity of reporter plasmids as they contain artificially combined TCF binding sites which does not reflect the endogenous circumstances. Additionally, reporter detects TCF/LEF mediated transcriptional activation, but β-catenin also exerts its transcriptional activation through other co-activators and these activations will not be detected with these constructs (Vincan, 2008).

In the light of our preliminary data, we hypothesized that SETD3 regulates mESC differentiation by regulating the canonical Wnt signaling pathway activity

and the differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium is caused by insufficient activation of the pathway. Overall, our results support this hypothesis. However, in their studies Lyashenko et al., suggested that cell adhesion function of β-catenin is more important than its transcriptional activity during endoderm differentiation, while mesoderm differentiation requires transcriptional function more than cell adhesion function. Yet, there were several weaknesses of the paper. In their studies they used C-terminus truncated β-catenin rescue (β-cat^{resΔC}) mESCs and suggested these cells are signaling defective. However, Helix-C region which extends from 667th aa to 683rd amino acid is important for the transcriptional function of β-catenin, but they deleted amino acid residues from 727 to 781 (Sustmann et al., 2008; Xing et al., 2008). Therefore, Helix-C region and the still transcriptional activity is still intact.

To show the signaling deficiency, they performed a luciferase assay, and no signal was observed in β-cat^{resΔC} mESCs. However, they also observed low reporter activity in full β-cat^{resWT} mESCs, even though higher than β-cat^{resΔC}. As they have also reported, Rosa26 transgene levels were very low. Probably transcriptional activity could not be detected as very low levels of the construct was not sufficient to participate in transcriptional activity of β-catenin. If promoter was to be stronger, maybe transcriptional activity would be also observed in β-cat^{resΔC} mESCs. Additionally, luciferase assay results were normalized. Even though there seems to be high fold difference between the reporter activity of wild type cells and C-terminus deleted β-catenin constructs, expression of both constructs might be very low and biologically insignificant, therefore rendering the observed significant difference inaccurate and exaggerated.

Later, they performed embryoid body differentiation by using LIF withdrawal method. Due to LIF withdrawal, spontaneous differentiation occurs instead of directed endoderm or mesoderm differentiation. They could have used a more specific differentiation protocol to study differentiation, as spontaneous differentiation results in differentiation to all three primary germ layers. They observed smaller colony sizes and more single cells in β-catenin deficient (β-cat$^{Δ/Δ}$)

mESCs at the 7th day of embryoid body differentiation. They suggested that this observation is due to adhesion defects resulting from the absence of β-catenin, but not from its transcriptional function, as β-cat$^{res\Delta C}$ mESCs showed similar properties to wild type mESCs. However, this observation is not enough to conclude that, as cell death might be increased, or loss of β-catenin might influence expression of some genes which leads to lower proliferation rate. Additionally, they based their conclusion to the fact that β-cat$^{res\Delta C}$ mESCs are signaling defective but this is not certain.

Contradicting Lyashenko et al findings, we have observed lower nuclear levels of β-catenin in setd3Δ mESC that are grown in standard serum-containing mESC medium, which shows an endoderm differentiation defect. Additionally, a significantly lower level of transcriptional activity was observed in setd3Δ mESCs regardless of their growth medium. Even though these results do not show direct evidence, they strongly suggest a role of canonical Wnt signaling activity in observed endoderm differentiation defect of setd3Δ mESCs grown in standard serum-containing mESC medium.

Overall, absence of SETD3 is observed to be affecting the nuclear levels of β-catenin in mESCs grown in standard serum-containing mESC medium. Additionally, its absence is observed to result in a significant decrease in canonical Wnt signaling pathway activity in mESCs, regardless of the growth medium. These findings support our hypothesis that differentiation defect in setd3Δ mESCs grown in standard serum-containing mESC medium might be due to lower levels of nuclear β-catenin and lower canonical Wnt signaling activity.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

SETD3 was initially identified as a histone lysine methyltransferase which dimethylates H3K4 and H3K36. Later, researchers found that it methylates histidine 73 of actin and further suggested that SETD3 is only performing this methylation but does not have a histone methyltransferase activity. However, our endoderm differentiation data and results of differentially expressed gene analysis suggest that SETD3 is regulating gene expression especially throughout mesendoderm differentiation. To check whether cytoplasmic function of SETD3 as an actin methyltransferase might have a role in the endoderm differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium, we checked the level of actin cytoskeleton in wild type, setd3Δ and rescue mESCs by performing a phalloidin staining. We observed similar actin cytoskeleton in three of these cell lines, suggesting that defect might be due to SETD3's nuclear function instead of its actin methylation function. Later, KEGG analysis results from our preliminary data were validated using GSEA and Wnt signaling pathway was consistently observed to be enriched by genes that are expressed more in wild type cells compared to setd3Δ cells at 2nd and 3rd days of endoderm differentiation.

Therefore, we aimed to understand the effect of SETD3 on β-catenin level, localization and the canonical Wnt pathway activity in mESCs grown in two different media (2i4 medium or standard serum-containing mESC medium). To validate a potential role of canonical Wnt signaling pathway, interaction of SETD3 and β-catenin, which is the key transcription factor of the canonical Wnt signaling pathway, was investigated by performing PLA assay. Interaction of SETD3 and β-catenin proteins were observed in mESCs either grown in standard serum-containing mESC medium or 2i4 medium. Even though non-canonical Wnt signaling pathways might also play a role in endoderm differentiation defect observed in setd3Δ mESCs

grown in standard serum-containing mESC medium, in line with our preliminary data and PLA results, we focused on the effect of SETD3 on canonical Wnt signaling pathway.

ICC assay was performed with wild type and setd3Δ mESCs grown either in 2i4 medium or standard serum-containing mESC medium to observe the effect of SETD3 protein on total β-catenin levels and localization. Results showed that mESCs grown in 2i4 medium had higher levels of β-catenin regardless of their SETD3 status and mESCs grown in same media had similar levels of β-catenin. Even though nuclear, cytoplasmic and plasma membrane localization of β-catenin was observed, a clear distinction about the nuclear signal could not be made. Western blot analysis results performed with whole cell lysates supported ICC assay results.

To further investigate localization differences of β-catenin, Western blot analysis was performed with nuclear and cytoplasmic fractions of mESCs. Both nuclear and cytoplasmic β-catenin levels were observed to be higher in mESCs grown in 2i4 medium regardless of their SETD3 status. Similar to ICC and whole cell Western blot analysis results, cytoplasmic β-catenin levels of mESCs grown in the same medium did not show any differences. However, setd3Δ mESCs grown in standard serum-containing mESC medium showed lower levels of nuclear β-catenin compared to wild type mESCs grown in this medium.

As lower levels of nuclear β-catenin suggest lower levels of canonical Wnt signaling activity, a dual luciferase assay was performed. Significantly higher TCF/LEF mediated canonical Wnt signaling activity was observed in mESCs grown in 2i4 medium compared to their counterparts grown in standard serum-containing mESC medium. Surprisingly, setd3Δ mESCs grown in 2i4 medium showed significantly lower TCF/LEF mediated canonical Wnt signaling pathway activity compared to wild type mESCs grown in this medium. It suggests that presence of SETD3 is still affecting the canonical Wnt signaling activity even though similar nuclear β-catenin levels were observed. Additionally, consistent with our hypothesis

setd3Δ mESCs grown in standard serum-containing mESC medium showed significantly lower canonical than other three mESCs.

In conclusion, presence of SETD3 is observed to be lowering the nuclear levels of β-catenin as well as the canonical Wnt signaling pathway activity. Even though these are supporting evidence, these results suggests that lower activity of canonical Wnt signaling pathway may have a role in the differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium.

This conclusion brings up many questions that needs further investigation such as where does SETD3, and β-catenin interact in the cell? In the future, to validate the findings of PLA, co-immunoprecipitation (coIP) experiment with whole cell lysates of wild type mESCs can be performed and SETD3 – β-catenin interaction can be confirmed. As SETD3 is known to function in the cytoplasm as an actin methyltransferase, and as a histone methyltransferase in the nucleus, interaction might be both in the nucleus or in the cytoplasm (Eom et al., 2011; Kim et al., 2011; Kwiatkowski et al., 2018). To address the location of SETD3 – β-catenin interaction, coimmunoprecipitation (coIP) can be repeated using nuclear and cytoplasmic fractions. If western blot results after nuclear coIP experiment show that they interact in the nucleus, this will further enhance the hypothesis that SETD3 which introduces activatory modifications like H3K36me2 and H3K4me2 might be interacting directly with β-catenin, or as a part of a protein complex, to activate the transcription of canonical Wnt pathway target genes. Interaction might be also observed in both compartments suggesting a regulatory interaction.

If they interact in the nucleus, is SETD3 also involved in the β-catenin/TCF/LEF complex which is bound to DNA to activate transcription? To examine SETD3-β-catenin-DNA interaction, sequential chromatin immunoprecipitation (ChIP) experiments can be performed using SETD3 and β-catenin antibodies. Mutual binding to canonical Wnt signaling pathway target gene promoters can be investigated with qPCR analysis using primers for the canonical Wnt signaling pathway target genes. If these two proteins interact inside the nucleus

and the interaction is on the DNA to activate transcription, we would expect to see enhanced immunoprecipitation of promotor regions of canonical Wnt signaling pathway target genes. Interaction of these two proteins on DNA can be also investigated by performing an electrophoretic mobility shift assay (EMSA). In EMSA, if they bind to DNA together, we would expect to see an upper shift of the group which contains both TCF/LEF, β-catenin and SETD3 compared to other groups.

SETD3 protein contains two domains one of which is a SET domain that is required for its catalytic activity, and the other is a Rubisco-LSMT domain which is involved in the substrate recognition (Witecka et al., 2021). To determine which domain of SETD3 is involved in its interaction with β-catenin, domain deleted SETD3 can be generated and stably transfected to setd3Δ mESCs grown in standard serum-containing mESC medium. Localization of SETD3 domain deleted mESCs can be observed with ICC assay. If localization is the same, interaction can be investigated performing PLA, if not, PLA results might be misleading. When the interaction domain is found, phalloidin levels can be investigated to make sure deletion or mutation of the responsible domain is not interfering with the cytoplasmic function of SETD3. Later, by mutating or deleting the responsible domain, endoderm differentiation can be performed. If interaction of SETD3 and β-catenin is the reason for the endoderm differentiation defect, same defect will also be observed in this case. Finally, to investigate the effect of interaction on canonical Wnt pathway activity, luciferase assay can be performed.

Another question that arises is how direct evidence of SETD3's effect on canonical Wnt signaling activity can be shown? We suggested that normal endoderm differentiation time course observed in setd3Δ mESCs grown in 2i4 medium is due to their ability to upregulate expression of target canonical Wnt signaling pathway genes to the same extend as wild type cells. Additionally, we observed that setd3Δ mESCs grown in standard serum-containing mESC medium had significantly lower TCF/LEF mediated transcriptional activity, meaning there is significantly lower expression of β-catenin target genes. Even though expression data about target genes

is found in RNA-seq results and expression levels of some Wnt signaling pathway target genes were validated by RT-qPCR analysis, within our previous TUBITAK 1001 project, expression of target genes needs further validation. These can be checked by performing RT-qPCR experiments using primers for canonical Wnt signaling pathway target genes, and expression levels in wild type or setd3Δ mESCs grown in either 2i4 or standard serum-containing mESC medium can be compared.

As we have suspected that endoderm differentiation defect observed in setd3Δ mESCs grown in standard serum-containing mESC medium might be due to lower transcriptional level of canonical Wnt signaling pathway, it can be further confirmed with administration of canonical Wnt signaling pathway inhibitors to the mESCs (Z. Liu et al., 2021). Wild type mESCs can be cultured in standard serum-containing mESC medium which also contains canonical Wnt signaling pathway inhibitors and endoderm differentiation can be repeated. If the observed defect in the absence of SETD3 is due to insufficient activation of the pathway, same defect will be also observed when canonical Wnt signaling pathway inhibitors are used.

Finally, how differentiation defect can be shown to result from SETD3's nuclear function instead of its cytoplasmic function as a actin histidine N-methyltransferase? There are some newly identified SETD3 inhibitors which bind to β-actin interacting residues and prevents its methylation (Hintzen et al., 2021). Amount of inhibitor can be adjusted so that activity of SETD3 in the cytoplasm is inhibited but there is remaining SETD3 that can go and function in the nucleus. Correct nuclear localization can be checked with ICC assay or with western blot experiments performed with nuclear and cytoplasmic fractions of mESCs. Through which residues does SETD3 and β-catenin interact and where does interaction occur is unknown. If interaction occurs in the cytoplasm and the same residues are used to interact with β-catenin, inhibitors will also abolish the interaction between SETD3 and β-catenin. Therefore, results will not be reliable. To ensure interaction is still intact PLA should be performed. If both of these properties are validated, endoderm differentiation experiments with mESCs grown in standard serum-containing mESC medium which are treated with these inhibitors can be performed. If endoderm

differentiation defect is not observed in wild type mESCs which are treated with SETD3 inhibitors, this will further support that defect is due to the nuclear function of SETD3. If nuclear function is responsible from the defect, we would also expect to see similar levels and localization of β-catenin in wild type mESCs grown in standard serum-containing mESC medium with or without SETD3. Also, similar levels of canonical Wnt signaling activity would be expected in luciferase assay.

Additionally, as we are suspecting from the nuclear function of SETD3 in the observed differentiation defect, a putative nuclear localization signal (NLS) can be found. Putative NLS can be deleted or mutated, and a stable cell line can be created, transfecting this construct to setd3Δ mESCs (setd3Δ-setd3ΔNLS mESCs). To validate SETD3 cannot localize to nucleus, ICC assay or western blot technique by using nuclear and cytoplasmic fractionations can be performed. If SETD3 is not able to translocate to nucleus in these cells, endoderm differentiation can be performed again using wild type, setd3Δ and setd3Δ-setd3ΔNLS mESCs grown in standard serum-containing mESC medium. If the nuclear function of SETD3 is responsible from this endoderm differentiation defect in setd3Δ mESCs, as we have hypothesized, we would see a similar endoderm differentiation defect in setd3Δ-setd3ΔNLS cells. By using setd3Δ-setd3ΔNLS mESCs PLA can be performed to observe whether the interaction of SETD3 and β-catenin is in the nucleus or not. Additionally, β-catenin levels and localization can be investigated following the same set of experiments used in this study and changes in transcriptional activity of β-catenin can be investigated with dual luciferase assay.

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APPENDICES

A. Medium Recipes used in Cell Culture

Table 3. MEF Medium Recipe for 100 mL

Reagent	***Required Volume***	***Brand/ Catalog Number***
DMEM	88 mL	Gibco, 41966029
Fetal Bovine Serum (FBS)	10 mL	Gibco, 10270106
GlutaMAX I	1 mL	Gibco, 35050061
Pen/Strep	1 mL	Gibco, 15140122

Table 4. Standard serum-containing mESC medium for 100 mL

Reagent	***Required Volume***	***Brand/ Catalog Number***
DMEM	80 mL	Gibco, 41966029
FBS	15 mL	Gibco, 10270106
Pen/Strep	2 mL	Gibco, 15140122
GlutaMAX I	1 mL	Gibco, 35050061
Nucleoside Mix	1 mL	*See* Solution Recipes (Appendix B)
MEM NEAA	1 mL	Gibco, 11140-035
β-mercaptoethanol (BME)	0.704 µL	Sigma, M-6250
Supplement for 100 mL		
Leukemia Inhibitory Factor (LIF)	1000 units/mL (10 µL)	Millipore, ESG1107

Table 5. 2i4 Medium Recipe for 100 mL

Reagent	*Required Volume*	*Brand/ Catalog Number*
DMEM/F12	50 mL	Gibco, 11320074
Neurobasal	50 mL	Gibco, 21103049
FBS	4 mL	Gibco, 10500064
50x B27 Supplement with Retinoic Acid	1 mL	Gibco, 35050061
Pen/Strep	1 mL	Gibco, 15140122
GlutaMAX I	1 mL	Gibco, 35050038
10% Bovine Serum Albumin (BSA) in PBS	500 µL	Sigma, A3311-50G
100x N2 Supplement	500 µL	Capricorn, N2-K
Monothioglycerol (MTG)	1.3 µL	Sigma, M6145-25ML
Supplements (for 100 mL)		
LIF	1000 units/mL (10 µL)	Millipore, ESG1107
CHIR99021	3 µM (30 µL)	Cayman, 13122-5mg
PD0325901	1 µM (10 µL)	Selleckchem, S1036-5mg

B. Solution Recipes

0.1% Gelatin Solution: 0.1g Gelatine from Bovine Skin (Cat. No.: G9391-100G, Sigma) was added to 100 mL Cell Culture Grade Water (Cat. No.: 03-055-1A, Biological Industries). It completely dissolves after autoclave. Stored at 4°C.

4% Paraformaldehyde (PFA): 16% PFA was prepared by dissolving 80g paraformaldehyde (Cat. No.: 104005, Merck) in 400 mL distilled water at 60°C. pH was adjusted to 7.0 and volume was completed to 500 mL with distilled water. It was aliquoted and stored at -20°C . 16% PFA was diluted with 1X PBS to prepare 4% PFA. Stored at 4°C.

0.5% Triton X-100: 10% Triton X-100 was prepared by mixing 10 mL Triton X-100 (Cat. No.: M143-1L, VWR) and 90 mL 1X PBS. Then, 0.5% Triton X-100 was prepared by mixing 5 mL of 10% Triton X-100 with 95 mL 1X PBS. To prepare 0.1% Triton X-100, 1 mL 10% Triton X-100 was mixed with 99 mL 1X PBS.

10% Bovine Serum Albumin (BSA): After dissolving 10 g Bovine Serum Albumin (Cat. No.: A3311-50G, Sigma) in 100 mL Dulbecco's Phosphate Buffered Saline (DPBS) (Cat. No.: 02-023-1A, Biological Industries) it was sterilized by filtering and stored at 4°C. 1% BSA solution was prepared by diluting 10% sterilized BSA with 1X PBS. Stored at 4°C.

2x Laemmli Sample Buffer Solution: 50 µL BME was added to 950 µL 2x Laemmli Sample Buffer (Cat. No.: 161-0737, Biorad). Prepared freshly.

4x Laemmli Sample Buffer Solution: 100 µL BME was added to 900 µL 4x Laemmli Sample Buffer (Cat. No.: 1610747, Biorad). Prepared freshly.

10X Running Buffer: 30 grams of Tris base (Cat. No.: M151-1KG, Amresco), 144 grams of Glycine (Cat. No.: SE2339004, Serva) and 10 grams of Dodecylsulfate Na-salt in pellets (SDS) (Cat. No.: SV0021, Serva) were dissolved in 500 mL distilled water and volume was completed to 1L with distilled water. Stored at RT.

Alternative Transfer Buffer: 5.6 grams of Tris base and 3 grams of Glycine were dissolved in 500 mL distilled water. 200 mL Methanol (Cat. No.: 348885-2.5L, Sigma) was added, and volume was completed to 1L using distilled water. Stored at 4°C.

20X TBS: 48.4 grams of Tris base and 160 grams of Sodium Chloride (NaCl) (Cat. No.: M106404.1000, Merck) were dissolved in 700 mL distilled water. pH was adjusted to 7.6 by using 37% Hidrochloric Acid (HCl) (Cat. No.: 100317, Merck) and volume was completed to 1L by using distilled water. Stored at RT.

10% Tween-20: 10 mL Tween-20 (Cat. No.: 0777-1L, VWR) was put into 100 mL distilled water. Stored at RT.

1X TBS-T: 50 mL 20X TBS and 10 mL 10% Tween-20 were mixed, and volume was completed to 1L by using distilled water. Stored at RT.

5% Skim Milk Solution: 5 grams of Skim Milk Powder (Cat. No.: 70166-500G, Sigma) was weighted and dissolved in 100 mL of 1X TBS. Prepared freshly.

Nucleoside Mix Solution: 80 mg Adenosine (Cat. No.: A4036-5G, Sigma), 73 mg Cytidine (Cat. No.: C4654-1G, Sigma), 85 mg Guanosine (Cat. No.: G5264-1G, Sigma), 24 mg Thymidine (Cat. No.: T1895-1G, Sigma), 73 mg Uridine (Cat. No.: U3003-5G, Sigma) were dissolved in 100 mL distilled water by heating to 45°C. Sterilized by using a filter while it was still warm and stored at -20°C.

C. Antibodies Used in the Experiments

Table 6. Antibodies Used in ICC assay and PLA

Protein Name	***Cat. No., Brand***	***Host***	***Dilution***
β-catenin	sc133240, Santa Cruz	Mouse	1:250
SETD3	NBP2-32136, Novus	Rabbit	1:250
Goat Anti-Rabbit IgG H&L (Alexa Fluor 594)	ab150080, Abcam	Goat	1:1000
Goat Anti-Mouse IgG H&L (Alexa Fluor 488)	ab150117, Abcam	Goat	1:1000

Table 7. Antibodies used in Western Blot Experiments

Protein Name	***Cat. No., Brand***	***Host***	***Dilution***
β-catenin	sc133240, Santa Cruz	Mouse	1:1000
GAPDH	2118S, Cell Signaling Technologies	Rabbit	1:3000
BRD2	d89b4, Cell Signaling Technologies	Rabbit	1:1000
HDAC1	06-720, Millipore	Rabbit	1:1000
Goat Anti-Mouse IgG H&L (HRP)	ab97023, Abcam	Goat	1:5000
Goat Anti-Rabbit IgG H&L (HRP)	ab97051, Abcam	Goat	1:5000

D. Wnt Signaling Pathway Overview

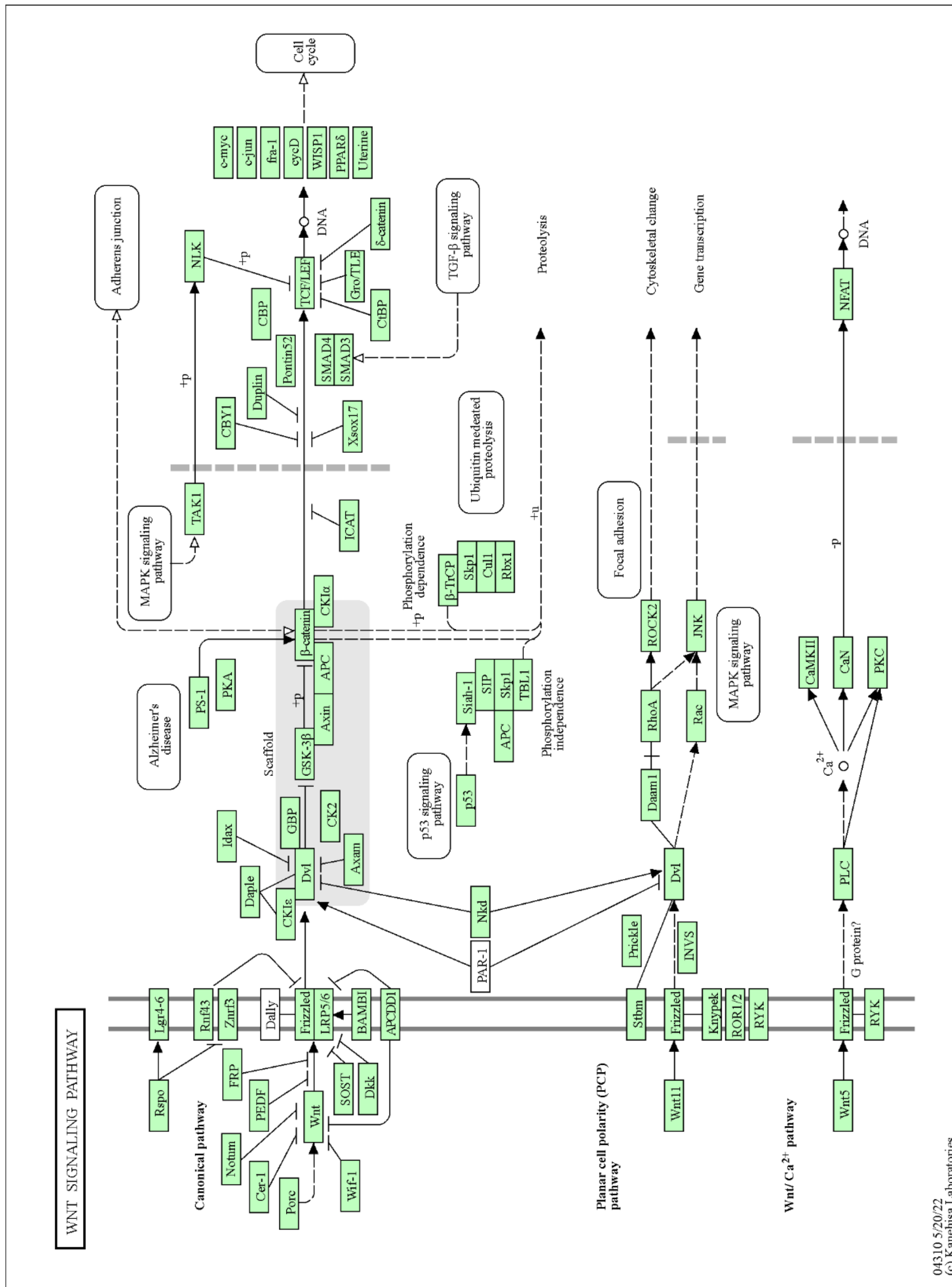

Figure 5.1. Overview of canonical and non-canonical Wnt signaling pathways (PCP and Wnt/Ca^{2+} pathways) in *Mus Musculus*. Derived from KEGG database.