Functional characterization and physiological significance of SETD3

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Abbreviations

Rubisco LSMT	Rubisco large subunit methyltransferase
LPS	Lipopolysaccharides
PTMs	Post translational modifications
H3	Histone 3
H4	Histone 4
HDAC	Histone deacetylases
MBP	Maltose binding protein
GTPase	Guanosine triphosphate hydrolase enzymes
KMT	Lysine-specific methyltransferase
PRMT	Protein arginine methyltransferase
SAM	S-adenosylmethionine
PRC2	Polycomb repressive complex 2
Me0	unmethylated
Me1	monomethylated
Me2	dimethylated
Me3	trimethylated
EZH2	Enhancer of zeste homolog 2
SETD3	SET domain containing 3
CrispR	Clustered regularly interspaced short palindromic repeats
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
GFP	Green fluorescent protein
WT	Wild type
MT	Mutant
kDa	Kilodalton
PCR	Polymerase chain reaction
BMDM	Bone marrow derived macrophages
BMDC	Bone marrow derived dendritic cells
KO	Knockout

Abstract

Lysine methyltransferase family plays important roles in various cellular processes, for instance transcriptional regulation, embryonic development, cell proliferation, migration and more. My project aims to characterize a novel SET-domain containing lysine methyltransferase SETD3 and understand the physiological significance of this protein. SET-domain containing protein SETD3 methylates Lys-4 and Lys-36 of histone 3, which is linked to the transcriptional activation. In addition, SETD3 also comprises of Rubisco LSM1 C-terminal substrate binding domain that could potentially facilitate binding to non-histone substrate. In our preliminary studies, we identified that SETD3 was able to methylate a 42kDa protein in the cytosolic fraction. According to the size estimation, we conducted SETD3 methylation assays with monomeric and polymeric form of actin. The result showed that monomeric and polymeric actin were the potent substrates of SETD3. From other studies, actin is shown to be methylated at Lys-84 and Lys-326 but the methyltransferases responsible for these methylations remain unknown. In the assays using actin mutants with Lys-84, Lys-326 or Lys-328 to alanine substitutions as substrate, SETD3 methylation activity was not reduced, suggesting that these lysines are unlikely to be the SETD3 target sites. In addition, SETD3 expression in B cells increased after the stimulation with anti-IgM antibodies, thymus-dependent signal CD40 and lipopolysaccharides (LPS). The preliminary flow cytometry immunophenotyping also revealed perturbed development of T cells, B cells, and neutrophils in the conditional SETD3-deficient mice (Mx-cre). These results suggest that SETD3 may play physiological roles in the development, activation, and effector functions of various immune cells. In future, the physiological function of SETD3 in the development of immune cells and immune responses will be further examined using our conditional knockout mouse model.

1. Introduction

The discovery of post translational modifications (PTMs) has unveiled the higher complexity of proteome network at least half a century ago (Burnett 1954, Ambler 1959). Immense numbers of PTMs have been studied structurally and functionally. Far from being “accessories” of proteins, PTMs play an essential role in numerous fundamental cellular behaviours including transcriptional regulation, signal transduction, cell division, and apoptosis (Gajewski 1996, Okajima 2002, Shogren-Knaak 2006, Li 2013). Generally, proteins are chemically modifiable by PTMs such as acetylation, methylation, phosphorylation, glycosylation and more (Gajewski 1996, Okajima 2002, Shogren-Knaak 2006, Li 2013).

1.1 Protein Phosphorylation

Various signalling pathways and cellular processes are regulated by phosphorylation including transcription, apoptosis, cytoskeleton arrangement, cell-cycle progression, immunological functions and more (Reviewed in Johnson 2009). Protein kinases transfer a single phosphate group from adenosine triphosphate (ATP) to hydroxyl group of Tyrosine (Tyr), Serine (Ser) or Threonine (Thr). Phosphatases mediate the process termed dephosphorylation that removes phosphate group from proteins (Reviewed in Zhang 2016). Combined effect of phosphorylation and dephosphorylation is crucial in response towards external stimuli, such as growth factors, cytokines and more (Witthuhn 1994, Heldin 1995, Mizuno 2013). Secondary messengers such as cAMP or Ca^{2+} regulate the activity of protein kinases and phosphatases. It has been reported that secondary messengers mostly associate with Ser/Thr kinases, whereas external stimuli (e.g. growth factors) activate Tyr kinases. At high level of secondary messengers, inhibitory subunit is out-competed and removed from the catalytic site of kinases, thereby activating these kinases (Purves 2001). At low level of cAMP, inhibitor for phosphatases is inactive leading to the activation of phosphatases (Lodish 2000). However, certain phosphatase such as calcineurin is activated in high level of cytoplasmic Ca^{2+} (Crabtree 2001).

1.2 Protein Acetylation

Protein acetylation has a fundamental function in regulating gene transcription. For instance, addition of acetyl group to the N-terminus of histones (lysine 5 of H2A; lysine 5, 12, 15 and 20 of H2B; lysine 4, 9, 14, 18, 23, 36 and 56 of H3; lysine 5, 8, 12 and 16 of H4) neutralizes the positive charge at the histone tails. Thereby, nucleosome conformation is altered which increases the accessibility of transcriptional regulatory proteins to DNA template (Reviewed in Zhang 2016). In addition, acetylation of P53 and GATA1 at the site adjacent to DNA binding domain resulted in better DNA binding (Gu 1997, Boyes J 1998). Acetylation has been documented in many different cytoplasmic processes as well, for instance autophagy, cytoskeleton reorganization, endocytosis and more (Matthias 2008). Lysine acetylation is a reversible process in both nucleus and cytoplasm, mainly catalysed by deacetylases (HDAC) leading to transcriptional repression and regulation of protein activity (Nan 1998, Matthias 2008). HDACs often function in complexes that are important to govern its substrate specificity. For instance, most of the class I HDAC are functioning in complex with NURD, SIN3a, and Co-REST (Yang 2008).

1.3 Protein Glycosylation

Glycosylation, the attachment of sugar residues, is denoted as the most complicated posttranslational modification among various cell types and species. There are at least 8 amino acids and 13 monosaccharides which participate in protein glycosylation (Reviewed in McCarthy 2014). Addition of N-Acetylglucosamine (GlcNAc) to the functional group of serine and threonine, commonly known as O-glycosylation, is the most common modification among various types of glycosylation (Reviewed in Spiro 2002). Some diseases have been reported to be caused by irregularity in the glycosylation, such as neurological and developmental deficiencies (Reviewed in Freeze 2001). Cellular glycosylation could also be affected by extracellular factor, for instance clostridial cytotoxins known as the causative agent for botulism, colitis, gas gangrene and diarrhea, as a result from the unnecessary addition of sugar group at Threonine residue of Rho family GTPases (Reviewed in Busch 2000). The antagonistic effect of such modification affects

the effector binding and thereby inhibits GTPase activity leading to signaling dysregulation (Reviewed in Busch 2000).

1.4 Protein Methylation

Protein methylation is mainly associated with lysine and arginine residues. The methyl group transfer to the ϵ -amino group of lysine may be mono-, di- or trimethyl group, mediated by lysine-specific methyltransferase (KMT) in the presence of methyl donor, S-adenosylmethionine (SAM). In contrast, arginine may only be mono- or dimethylated by arginine methyltransferases (PRMTs) in the presence of SAM (Grillo 2005). Unlike the well-studied protein phosphorylation, protein methylation remains a nascent field, mainly due to limited proteomic techniques that are suitable for analysis of this modification. Some technical advances have been established for the analysis of histone methylation, however this is frequently challenging for the analysis of less abundant proteins (Cao 2016).

1.5 Lysine Methyltransferase - SET Domain Containing Protein

In most cases, site-specific lysine methylation is catalyzed by SET-domain-containing protein. SET domain is originally identified as an evolutionary conserved region in drosophila suppressor of variegation 3–9, enhancer of zeste and trithorax group proteins (Jones RS 1993, Tschiersch 1994, Stassen 1995). Based on the motif sequence similarity, lysine methyltransferases are categorized into seven families, namely EZ, SUV39, SET1, SET2, SMYD, RIZ and SUV4-20 (Reviewed in Dillon 2005). All lysine methyltransferases carry the conserved SET domain except DOT-1 (also called DOT-1 like protein (DOT1/DOT1L)), which specifically methylates H3 Lys-79 (Feng 2002).

Lysine methyltransferase family of proteins play important roles in various physiological and pathological conditions. SET-domain-containing proteins such as SUV39H1, SUV39H2, and SET9 functioning as H3 or H4 methyltransferases are critical epigenetic regulators in mammalian development, genomic instability and heterochromatin (Sanders 2004, Peters 2001). Overexpression of SET-domain-containing proteins or loss of SET-domain in some of these proteins is associated with cancers. For instance, mixed lineage

leukemia 1 (MLL1) turns oncogenic when SET domain is lacking due to chromosomal translocations (Ayton 2001). On the other hand, overexpression of Ezh2 (SET-domain-containing methyltransferase) is associated with aggressive cancer in prostate, breast, bladder, gastric, lung and hepatocellular carcinoma (Varambally 2002, Kleer 2003, Arisan 2005, Sudo 2005, Matsukawa 2006, Watanabe 2008). Other SET-domain-containing proteins such as NSD3 and SMYD3 are amplified in the breast cancers (Angrand 2001, Hamamoto 2006). Uncontrolled cell proliferation is also induced by the binding of Sbf-1 to the SET domain of SUV39H1 (Reviewed in Schneider 2002). Truncated SETD3 is found to be associated with oncogenesis which is discussed further in section 1.10.

1.6 Lysine Methylation on Histone Protein

Histone methylation leads to changes in the physical properties of chromatin and regulates gene expression through recruitment of specific transcriptional regulators. Lysine residues namely Lys-4, Lys-9, Lys-27, Lys-36 and Lys-79 of histone H3 as well as Lys-20 of histone H4, are the primary targets for histone methyltransferases (Reviewed in Kouzarides 2007). Varying degree and location of lysine methylation will result in two outcomes, which can be activation or repression for gene expression. For instance, genes associated with histone H3 Lys-9, H3 Lys-27 or H4 Lys-20 methylation are frequently repressed, whereas genes marked by H3 Lys-4, H3 Lys-36, and H3 Lys-79 methylation are activated (Reviewed in Zhang 2012).

Study has shown the association of H3 Lys-9 trimethylation with heterochromatin protein HP1 contributes to the formation of heterochromatin structure (Rice 2003). However, H3 Lys-9 methylation is not only restricted to heterochromatic region. Mono- and dimethylation, have been identified at the euchromatic regions and associated with temporal gene repression (Rice 2003). This regulatory mechanism is mainly catalyzed by G9a or G9a-related protein (GLP) (Rice 2003). Deficiency of these histone methyltransferases result in severe reduction of H3 Lys-9 methylation in mice leading to up regulation of various genes (Tachibana 2002). Additionally, SUV39H1 and SUV39H2 have the ability to silence specific genes located at euchromatic region as well. These

lysine methyltransferases could be recruited to the promoters of S-phase genes and repress the gene expression (Nielsen 2001).

H3 Lys-27 methylation is another epigenetic marker that is known for its repressive feature. Trimethylation at H3 Lys-27 is associated with transcriptionally inactive chromatin, similar to H3 Lys-9 trimethylation (Reviewed in Grewal 2003). Its regulatory feature is catalysed by polycomb repressive complex 2 (PRC2), mainly occurs at facultative heterochromatin. However, H3 Lys-9 trimethylation is often associated with constitutive heterochromatin at telomeres and centromeres (Reviewed in Saksouk 2015). Several studies have identified the functions of H3 Lys-27 in mammalian cells such as X-chromosome inactivation, germline development, maintaining stem cell pluripotency and also participate in cellular proliferation (Reviewed in Cao 2004).

Methylation of H4 Lys-20 has functions in the DNA damage response, cell cycle regulation, DNA replication and chromatin compaction. Monomethylation of H4 Lys-20 is mainly catalysed by PR-Set7 or Set8, whereas Suv4-20 mediates trimethylation at the same amino acid (Reviewed in van Nuland 2016). Study has reported that H4 Lys-20 monomethylation is associated with chromosomal regions containing genes that need to be repressed in early mitosis (Karachentsev 2005). On the other hand, a study shows monomethylation at H4 Lys-20 could play an important role in transcriptional activation, for instance promoting the expression of Wnt reporter gene (Li 2011). The functional effects of such methylation is governed by the reader domains within a spatial and temporal context (Reviewed in van Nuland 2016). The concept of methylation readers is further discussed in section 1.8.

H3 Lys-4 methylation is generally associated with the activation of transcription. Trimethylation of H3 Lys-4 is commonly found to associate with 5' ends of ORF when genes expression are induced (Reviewed in Li 2007). Furthermore, H3 Lys-4 methylation allows the recognition of other proteins involved with transcriptional activation. In mammalian system, Chd1 interacts firmly with H3 Lys-4 methylation and regulates transcript elongation as well as pre-mRNA processing (Sims 2007). In contrast, recent study has discovered that monomethylation of H3 Lys-4 mediated by MLL3/4 marks the

promoters of repressed gene in embryonic fibroblast, macrophages and human embryonic stem cells. Monomethylation at H3 Lys-4 restrict the binding of ING-1 to the gene regions leading to gene repression (Cheng 2014).

H3 Lys-36 methylation is also recognized as marker for active transcription, which mainly found at the 3' end of active genes. In particular, the methylation is found interacting with the elongating form of RNA polymerase II (Krogan 2003). H3 Lys-36 also carries suppressive function, which is important to prevent inappropriate initiation during gene transcription (Carrozza 2005). Methylation at H3 Lys-36 attracts EAF3 protein, which then recruits Rpd35 deacetylase complex to the coding region, leading to subsequent deacetylation of associated chromatin (Carrozza 2005).

H3 Lys-79 is strongly correlated with active gene transcription, impairment of telomeric silencing, cell-cycle regulation and DNA repair. For instance, DOT1/DOT1L interacts with elongation factors and methylate H3-Lys79 to maintain active transcription (Nguyen 2011). The pathological role of H3 Lys-79 methylation in leukaemia is characterized (Reviewed in Farooq 2016). A number of MLL1 fusion chimeras have been identified to be associated with DOT1/DOT1L that caused hypermethylation of H3 Lys-79 leading to abnormal transcriptional activation (Okada 2005). In addition to the regulatory functions on histones, Lysine methylation of non-histone proteins has recently been developed into a popular research topic, which has significant regulatory roles in various cellular processes. This is further discussed in the next section.

1.7 Lysine Methylation on Non-Histone Proteins

Lately, studies of lysine methylation on non-histone proteins have emerged as one of the important PTMs regulating transcriptional activity. p53 is probably the best characterized lysine methylated protein that is identified a decade ago (Chuikov 2004). SET7/9 catalyses Lys-372 methylation of p53 in the regulatory region of C-terminus. Such methylation occurs when the cells are exposed to DNA damage leading to the activation of downstream protein p21 and initiation of cell cycle arrest (Ivanov 2007). Methylation of p53 also promotes p53 acetylation. Overexpression of SET7/9 results in high level of p53

acetylation (Ivanov 2007). In addition, several other non-histone substrates are identified over the years, such as NF- κ B, STAT3, DNMT1, FOXO3, HSP90, actin, talin and more. Table 1.7.1 summarizes the non-histone substrates, their corresponding lysine methyltransferases and the effects of methylation.

Non-histone substrate	Enzyme	Methylation site	Effect	Reference
Transcription				
p53	G9a/GLP	K373me2	Interrupt apoptotic activity	(Huang 2010)
	SMYD2	K370me1	Inhibit p53 function	(Huang 2006)
	SET8	K382me1	Repress p53 activity and downstream target gene expression	(Shi 2007)
	SET7/9	K372me1	Stabilizes p53 Promote p53 acetylation	(Ivanov 2007)
C/EBP β	G9a	K39	Inhibits transactivation	(Pless 2008)
Reptin	G9a	K67me1	Repress hypoxia related gene expression	(Lee 2010)
WIZ	G9a	K305me3	Promote binding of HP1 <i>in vitro</i>	(Rathert 2008)
GATA4	EZH2(PRC2)	K299me1	Inhibit interaction with p300 and downstream gene expression	(He 2012)
STAT3	SET7/9	K140me2	Activation or inhibition of certain target genes	(Yang 2010)
	EZH2	K180me3	Activation of STAT3 signalling	(Kim 2013)
		K49me2	Activate STAT3 in response to IL-6	(Dasgupta 2015)
ROR α	EZH2	K38me1	Induce proteasome degradation	(Lee 2012)
FOXO3	SET7/9	K270me1	Inhibit transactivation and neuronal apoptosis related to stress	(Xie 2012)
VEGFR	SMYD3	K831me2	Increase kinase activity	(Kunizaki 2007)
MyoD	G9a	K104me2	Inhibit myogenic differentiation	(Ling 2012)
NF- κ B	SET7/9	K37me1	Reduced gene expression in TNF α stimulation; promote p65 DNA binding	(Ea 2009)

Methyltransferases				
DNMT1	SET7/9	K142me1	Promote DNMT1 degradation	(Estève 2009)
DNMT3	G9a	K44me2	Interacts with MPP8	(Chang 2011)
G9a	G9a	K165me2/3	Recruits HP1	(Sampath 2007)
		K239me3	Promote binding with HP1	(Chin 2007)
SUV39H1	SET7/9	K105/K123me1	Response towards DNA damage; negatively regulate SUV39H1 activity	(Wang 2013)
GLP	GLP	K205	Binds with MPP8	(Chang 2011)
Acetyltransferases/Deacetylases				
HDAC1	G9a	K432	Interact with HP1 <i>in vitro</i>	(Rathert 2008)
CDYL1	G9a	K135me3	Blocks the binding to H3K9me3	(Rathert 2008)
Kinases/Phosphatases				
DNA-PKcs	-	K1150me2/3 K2746me3 K3284me3	Interacts with HP1 during DNA damage response	(Liu 2013)
Chaperones				
HSP90	SMYD2	K616me1	Promote binding to titin	(Voelkel 2013)
Cytoskeletons				
Actin	-	K84me1	Promote ALKBH4 and actomyosin interaction	(Li 2013)
		K326me2	-	(Vandekerckhove 1984)
Nopp140	-	K80me3	HP1 β interaction	(Liu 2013)
XIRP2	-	K1877me3 K1944me3	HP1 β interaction	(Liu 2013)
LAP2A	-	K94me3	HP1 β interaction	(Liu 2013)
Talin	EZH2	K2454me3	Inhibit binding of Talin to F-actin	(Gunawan 2015)

Table 1.7. 1 Summary of the non-histone substrate of lysine methyltransferase, methylation site and the effect of methylation

1.8 Readers of Lysine Methylation

PTMs serve as signal to recruit the effectors/readers to the modified chromatin. Lysine methyltransferase marks histone in four different states: unmethylated (me0), mono-(me1), di-(me2) and tri- (me3) methylation that are specifically recognized by various readers (Reviewed in Yun 2011). These chromatin readers are identified with multiple domains are identified in chromatin readers such as Tudor, double/tandem Tudor, MBT, PHD, chromo,

WD40, Ankyrin Repeats and more (Reviewed in Yun 2011). Methylation possesses strong site-specificity and the domains that recognize the same markers are found to be structurally similar (Reviewed in Yun 2011). Chromatin readers interact specifically at its preferred methylation site, for instance ING2 for H3 Lys-4, HP1 for H3 Lys-9, EED for H3 Lys-27, MRG15 for H3 Lys-36, PHF20 for H3 Lys-79, Crb2 for H4 Lys-20 and more (Rice 2003, Kim 2006, Zhang 2006, Wang 2009, Xu 2010, Cheng 2014). In certain instances, the recognition of different methyl states attracts different sets of effectors which determine the downstream functional effect. H4 Lys-20 me1 recruits the Pdp1 for cell-cycle regulation, whereas Crb2 binds to H4 Lys-20 me2 to regulate DNA damage checkpoint (Reviewed in Wang 2009). Different methyl states can also affect the binding strength for the same chromatin readers. For example, Rpd3S binds weakly to the Lys-36 me1 nucleosomes but binding affinity increases towards higher methyl-states (Li 2009). The methyl signal can be translated into many functional outcomes, such as recruitment of chromatin architectural proteins, chromatin remodelers, chromatin modifiers, transcription factors, repair machinery, RNA processing and replications (Reviewed in Yun 2011). In addition, several non-histone methyl readers are identified. For instance, DCAF1 is a monomethyl-specific reader that recognizes the ROR α (Lee 2012). TIP60 acetyltransferase recognizes methylated p53 to facilitate subsequent p53 acetylation (Kurash 2008).

1.9 Self-Methylation of Protein Methyltransferase

Self-catalytic feature of lysine methyltransferase is discovered about a decade ago. Most lysine methyltransferases are observed to be methylated, for instance lysine methyltransferases G9a, MLL1 and EZH2 (Chin 2007, Gunawan 2015) as well as arginine methyltransferases PRMT2, PRMT6 and PMRT8 (Dillon 2013, Sun 2014). The methylation of methyltransferases is proposed to have regulatory effects (Chin 2007, Dillon 2013). For instance, methylation of PRMT8 is induced by the deprivation of unmethylated substrates. Self-methylation of PRMT8 down regulates its own methylation activity by weakening its binding to methyl group donor (Dillon 2013). Methylation of G9a occurs at Lys-329 which mimics the consensus motif (ARKT) of H3 tail and regulates the binding of HP1 to G9a (Chin 2007).

1.10 SETD3 Lysine Methyltransferase

SETD3 is known as a transcription regulator that methylates H3 Lys-4 and Lys-36 (Eom 2011). Highly-conserved SET domain is located at N-terminus of SETD3 and RuBisCo lysine methyltransferase (LSMT) substrate binding-domain is found at the C-terminus of SETD3 (Chen 2012). SETD3 was proven to have function in muscle cell differentiation. Overexpression of SETD3 induces transcription of muscle-related genes, such as myogenic factor (Myf6), myogenin, and muscle creatine kinase (MCK) that stimulate muscle cell differentiation (Eom 2011). SETD3 is also identified as the interaction partner of proliferating cell nuclear antigen (PCNA). PCNA is an active recruiter of DNA replication enzyme to the site of DNA synthesis (Cooper 2015). This finding suggests that SETD3 might be involved in regulating DNA replication and repair processes. In a recent study, SETD3 was shown to interact with and methylate transcription factor FoxM1 and as a result inhibiting VEGF expression under normoxic conditions. In other words, under hypoxic conditions when VEGF expression is necessary, the interaction between SETD3 and FoxM1 will be weakened (Cohn 2016).

Rubisco LSMT C-terminal substrate binding domain is first characterized in Rubisco LSMT (Trievel 2003). Rubisco LSMT is a protein found in chloroplast, which shares the same domain architecture as SETD3, and methylates the large subunit of Rubisco (Houtz 1992). In addition, ribosomal lysine methyltransferase (RKM) 1 also contains the Rubisco LSMT C-terminal substrate binding domain, which shows methylation activity on ribosomal protein Rpl23ab (Porrás-Yakushi 2005). Judging from these findings, it is possible that SETD3 may have non-histone protein substrates in the cytosolic compartment.

SETD3 could be oncogenic in the absence of SET domain, which was resulted from chromosomal translocation between SETD3 at Chr12 and the Ig λ locus at Chr16. The translocation causes the formation of truncated SETD3, which is found to be overexpressed in CXP lymphoma as compared to normal tissues (Chen 2012). CXP lymphoma is a B lineage lymphoma arise from incomplete V(D)J recombination in Xrcc4/p53–double-deficient mice (Wang 2008). The mechanisms underlying this oncogenic activity of truncated SETD3 are yet to be determined. SETD3 is also found to be highly expressed in

renal cell tumors (RCTs) and chromophobe renal cell carcinoma (chRCC) (Pires-Luís 2015). Whether differential expression of SETD3, like other lysine methyltransferases can serve as the biomarker for cancer diagnosis, still remained to be determined (Pires-Luís 2015).

Up to date, most studies related to SETD3 are focused on functional implications of SETD3 as a histone methyltransferase. However, our studies indicate that SETD3 is a predominantly cytosolic protein in some cell types including BOSC23 cells (Figure 1.10.1) and immune cells (Bunjamin et al. unpublished data). Furthermore, immunofluorescence staining shown in human protein atlas reveal a co-localization of cytosolic SETD3 and mitochondria in several human cancer cell lines such as glioblastoma U-251 and osteosarcoma U-2 OS (<http://www.proteinatlas.org>). These data could indicate that SETD3 may regulate cellular activities in the cytosolic compartment. Epigenetic modification confers plasticity in the immune cell precursors during their differentiation and development as well as the effector functions of the mature cells (Kondilis-Mangum 2013). According to the data obtained from Immunological Genome Project (ImmGen), SETD3 expression level is considerably high in multiple cell types, including pro-B, pre-B, germinal center (GC) B cell, dendritic cell, plasmacytoid dendritic cell, neutrophil, CD4 T cell, CD8 T cell, and $\gamma\delta$ T cell (Figure 1.10.2). Even though changes in gene expression pattern do not always indicate critical roles of a protein in these cell types, it is still possible that SETD3 plays functional roles in these immune cells. To test our hypothesis, multiple experiments can be conducted, such as biochemical functional assays and immune profiling of SETD3 knockout mice model (Details see results section 3.4).

Figure 1.10. 1 Cytoplasmic localization of SETD3 in BOSC23 cells

SETD3-GFP fusion protein was transiently expressed in BOSC 23 cells. The GFP signal was detected by phase-contrast fluorescence microscope in live cells. Scale bar, 20 μ m. This data was generated by former lab members.

Figure 1.10. 2 mRNA expression level of SETD3 in characterized immunological cell lineage

Data are obtained from Immunology Genome Project (ImmGen): <http://www.immgen.org/databrowser/>

1.11 Research Objectives

SETD3-mediated methylation in the nucleus has been discussed, particularly with regards to the muscle cell differentiation, DNA replication or repair processes, and regulating transcriptional events under stress (Eom 2011, Cooper 2015, Cohn 2016). SETD3 is also associated with oncogenesis and could be used as the biomarker for cancer diagnosis (Pires-Luís 2015). However, these studies are very limited and lack comprehensive view about SETD3's functions in different cellular context, specifically in the regulation of cell activities and oncogenesis. On top of that, we are more interested in the behavior of SETD3 in the dynamics of immune responses, as SETD3 is found to be highly expressed in various immune cells (Figure 1.10.2) and enriched in the cytoplasmic compartment of macrophages (Bunjamin et.al, unpublished data). SETD3 is shown to be a H3K36 methyltransferase in the nucleus, its function in the cytosol remains to be determined. In this research project, our main objectives are:

- To identify the novel cytosolic substrates of SETD3 and the site of methylation through mutagenesis and mass spectrometry analyses
- To determine the physiological significance of SETD3-mediated methylation
- To characterize the self-methylation of SETD3 and its functional implications
- Immune profiling of the SETD3 conditional knockout mice to reveal the regulatory effects of SETD3 on immune cells

2. Materials and Methods

2.1 *In Vitro* Methylation Assays

25 µl of reaction mixture was prepared containing purified maltose-binding protein tagged SETD3 (MBP-SETD3), recombinant histone H3, 100 nci of [methyl-3H]-S-Adenosyl]-L-Methionine ([3H]-SAM) (PerkinElmer) in methylation buffer (50mM Tris [pH 8.5], 20mM KCl, 10mM MgCl₂, 10mM beta-mercaptoethanol, 250mM sucrose). The assays were performed overnight at 30°C. The reactions were terminated by 6x SDS-page dye and incubated at 95°C for 5 minutes. The samples were loaded and separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Staining of gels were done with Comassie Brilliant Blue R-250 to visualize the loaded samples, and then destained. The destained gels were incubated with 1x EN3HANCE (Perkin Elmer) for an hour followed by washing with ice cold water and subsequently gel drying. The dried gels were incubated with Kodak film for 5-10 days at -80 °C followed by detection of radioactive signal using imaging analyzer. For protein methylation assay, 3µg of cell lysate was added instead of histone H3. For SETD3-mediated actin methylation assay, 1µg/5µg of G-actin/F-actin (Cytoskeleton; ALK99) was used instead of histone H3. Automethylation assays were conducted with similar experiment setup in the absence of histone H3 and actin.

2.2 Polymerization of Purified G-actin

Actin polymerization procedure was conducted according to the datasheet of the product (Cytoskeleton; ALK99). G-actin stocks were reconstituted to recommended concentration and aliquoted in 10 µL volume. For actin polymerization, 1× general actin buffer (5mM Tris-HCl PH8.0 and 0.2mM CaCl₂) was added up to 10 times of the stock volume. The actin samples were incubated on ice for 30 minutes. After incubation, 10× actin polymerization buffers (500mM KCl, 20mM MgCl₂, 10mM ATP) was added in 1:10 ratio (by volume) and mixed followed by incubation at room temperature for 60 minutes. The polymerized actin (F-actin) can then be kept at 4°C for 6 months.

2.3 Recombinant Protein Expression and Purification

pTB-MAL vectors with SETD3 cDNA sequence (sequence id: [BC019973.1](#)) were inserted into *Escherichia Coli* strain BL21 via heat-shock transformation, followed by 1mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) (SIGMA) induction overnight at 18 °C. Amylose resins were used to purify induced MBP-SETD3 fusion proteins in accordance to manufacturer's protocol (pMAL™ Protein Fusion. & Purification System, New England Biolabs, NEB). Purified recombinant MBP-SETD3 proteins were used for in vitro protein methyltransferase assay.

2.4 Actin Lysine Site Directed Mutagenesis

Quikchange II XL site directed mutagenesis kit was used, and sample reactions were prepared according to the standard guidelines. The primers used in the site directed mutagenesis were included in table 2.4.1. The cycle of PCR was set according to table 2.4.2.

Lysine mutation	Primers
K50A	F 5'- GCCCACATAGGAATCCGCCTGACCCATGCCACC -3' R 5'- GGTGGGCATGGGTCAGGCGGATTCCTATGTGGGC -3'
K84A	F 5'-GGGACGACATGGAGGCAATCTGGCACCACAC-3' R5'- GTGTGGTGCCAGATTGCCTCCATGTCGTCCC-3'
K118A	F 5'- CATGATCTGGGTCATCGCCTCGCGTTGGCCTTG -3' R 5'- CAAGGCCAACC GCGAGGCGATGACCCAGATCATG -3'
K191A	F 5'- GCGCTCGGTGAGGATCGCCATGAGGTAGTCAGTC -3' R 5'- GACTGACTACCTCATGGCGATCCTCACCGAGCGC -3'
K237A	F 5'- GCAGCTCGTAGCTCGCCTCCAGGGAGGAGC -3' R 5'- GCTCCTCCCTGGAGGCGAGCTACGAGCTGC -3'
K290A	F 5'- GTTGGCGTACAGGTCTGCGCGGATGTCCACGTC -3' R 5'- GACGTGGACATCCGCGCAGACCTGTACGCCAAC -3'
K326A	F 5'- CTGGCACCCAGCACAATGGCCATCAAGATCATTGCTCC-3' R 5'-GGAGCAATGATCTTGATGGCCATTGTGCTGGGTGCCAG-3'
K328A	F 5'-CCCAGCACAATGAAGATCGCCATCATTGCTCCTCCTGAG-3' R 5'CTCAGGAGGAGCAATGATGGCGATCTTCATTGTGCTGGG-3'
K335A	F 5'- CCACACGGAGTACGCGCGCTCAGGAGGAGC -3' R 5'- GCTCCTCCTGAGCGCGCTACTCCGTGTGG -3'
K372A	F 5'- GGATCCTTAGAAGCATGCGCGGTGGACGATGGAG -3' R 5'- CTCCATCGTCCACCGCGCATGCTTCTAAGGATCC -3'

Table 2.4. 1 Primers for actin lysine site directed mutagenesis

Segment	Cycle	Temperature (°C)	Time (minute)
1	1	95	1
2	18	95	1
		60	1
		68	1min/kb of plasmid length
3	1	68	10
4	1	15	∞

Table 2.4. 2 PCR cycle for site directed mutagenesis of 3xFLAG-actin

2.5 Immunoprecipitation of 3x FLAG-Actin

BOSC23 cells were transfected with pCMV10/3xFLAG-actin, by using calcium phosphate transfection method. Same number of transfected cells (pull-down) and non-transfected cells (control) were lysed with hypotonic lysis buffer (10mM KCl, 20mM Hepes(pH7.9), 1mM EDTA, 1mM DTT, 1mM protease inhibitor cocktail (Roche) and 0.1% NP40) upon 48 hours transfection. After collecting cell lysate, salt concentration was adjusted to 150mM NaCl. Cytoplasmic lysates from both samples were pre-cleared with 15μL of equilibrated protein G sepharose beads (Amersham Biosciences) for at least an hour at 4 °C. Pre-cleared cell lysates were incubated with anti-FLAG antibody (12μL of antibody for 1mg/mL of protein lysate) at 4 °C overnight. Next, 75μL of protein G sepharose beads were added into both samples and incubated for another 4 hours at 4°C. After incubation, beads were washed with wash buffer (10mM KCl, 20mM Hepes(pH7.9), 1mM EDTA, 1mM DTT, 1mM protease inhibitor cocktail (Roche), and 150mM NaCl), followed by elution of 3xFLAG-actins with FLAG® tag peptides. The eluted 3xFLAG-actin were used for *in vitro* methylation assay.

2.6 B Cell Purification and *In Vitro* Stimulation

Spleens from wild type C57BL/6J were obtained, and mashed in between two pieces of 100 μM cell strainers in petri dish containing ice-cold Roswell Park Memorial Institute/Fetal Bovine Serum (RPMI/FBS) or Balanced Salt Solution/Fetal Bovine Serum (BSS/FBS). The cell suspension was then transferred into 15 mL tube and the cell strainers were washed with ice-cold RPMI/BSS or BSS/FBS again. The remaining cell suspension was collected and transferred to the same 15 mL tube. Following that, the cell suspension was spun down

at 453×g for 5 minutes in 4°C and supernatant was removed after centrifuged. Pellet was re-suspended with 1mL red blood cells (RBC) lysis buffer (9 parts of 155mM ammonium chloride to 1 part of 130mM Tris (hydroxymethyl) aminomethane) and incubated for 3 to 4 minutes at room temperature (RT). RBC lysis was stopped with 14mL ice-cold BSS/FBS and spun down at 453×g for 5 minutes in 4°C. The cell pellet was re-suspended in 300μl BSS/FBS up to 10⁸ splenic cells followed by incubation with 50μl anti-CD43 magnetic microbeads and 30μl of Annexin V magnetic microbeads for 30 minutes at 4°C. After incubation, the magnetic labeled cells were then washed with 14ml BSS/FBS and spun down at 453×g for 5 minutes in 4°C. Supernatant was removed and cell pellet was re-suspended in 1 to 3 ml of RT BSS. Cell suspension was transferred to equilibrated MACS column and flow-through containing B cells was collected. For in vitro stimulation, 24 well plates were added with 5μg/ml of F(ab')₂ goat anti-mouse IgM, 2μg/mL of anti-mouse CD40 monoclonal antibodies (mAbs) or both. In addition, well plates were also added with 20μg/ml of LPS for B cell stimulation. Purified B cells were cultured in these wells containing B cell stimulants at 37°C overnight. After stimulation overnight, B cells were harvested and fractionated for western blotting. A portion of the cell was used for flow cytometry analysis, to check for the up regulation of activation marker CD86.

2.7 Bone Marrow Derived Dendritic Cell (BMDC) Culture and *In Vitro* Stimulation

Major leg bones were obtained from wildtype C57BL/6J mice and bone marrow was flushed out by injecting RPMI medium. Cells were collected in 15ml tube and spun down at 453×g for 5 minutes at 4°C. Pellet was re-suspended with 1ml RBC lysis buffer (9 parts of 155mM ammonium chloride to 1 part of 130mM Tris (hydroxymethyl) aminomethane) and incubated for 3 to 4 minutes at room temperature. RBC lysis was stopped with 14ml ice-cold BSS/FBS and spun down at 453×g for 5 minutes at 4°C. The pellet was re-suspended in 10ml complete RPMI medium and plated on 10cm cell culture plate for 1 hour at 37°C. After incubation, the supernatant was collected and cell number was counted. Following that, 1 x 10⁶ cells were seeded in 24-well plate with medium containing 1:500 GMCSF and 1:1000 IL-4. At Day 2, half of the RPMI medium was replaced with fresh medium containing 1:1000 GMCSF. At day 4 onwards, growth medium was completely

replaced with fresh medium containing 1:1000 GMCSF. At day 7, BMDC were stimulated with 1 μ g/ml of LPS. Stimulated BMDCs were harvested for western blotting and flow cytometry analysis.

2.8 Bone Marrow Derived Macrophage (BMDM) Culture and *In Vitro* Stimulation

Major leg bones were obtained from wildtype C57BL/6J mice and bone marrow was flushed out by injecting RPMI medium. Cells were collected in 15ml tube and spun down at 453 $\times$ g for 5 minutes at 4°C. Pellet was re-suspended with 1ml RBC lysis buffer (9 parts of 155mM ammonium chloride to 1 part of 130mM Tris (hydroxymethyl) aminomethane) and incubated for 3 to 4 minutes at room temperature. RBC lysis was stopped with 14ml ice-cold BSS/FBS and spun down at 453 $\times$ g for 5 minutes at 4°C. The bone marrow cells were cultured in DMEM medium containing 20% FCS, 1% Pen/Strep, 1% L-Glutamine, 500 μ l Sodium Pyruvate, 10% L929 conditioned media. At day 6, BMDMs were stimulated with 100ng/ml LPS. After overnight LPS stimulation, both unstimulated and LPS stimulated mature BMDMs were obtained for western blotting and flow cytometry analysis.

2.9 SETD3-GFP Pull-Down

Approximately 6 $\times$ 10⁶ BOSC23 cells were transfected with pEGFP-SETD3. These cells were harvested and lysed in lysis buffer (10mM Tris/Cl pH7.5, 150mM NaCl, 0.5mM EDTA and 0.5% NP-40). The lysis process was conducted on ice with extensive pipetting every 10 minutes for half an hour. Cell lysate were spun down at 20000 $\times$ g for 10 minutes at 4°C. Lysate supernatant was transferred to a new tube. Volume of supernatant was adjusted to 500 μ L with ice cold dilution buffer (10mM Tris/Cl pH7.5, 150mM NaCl and 0.5mM EDTA). 25 μ L of GFP-Trap[®]_A beads were equilibrated in 500 μ L of dilution buffer followed by centrifugation at 2500 $\times$ g for 2 minutes at 4°C. Supernatant was discarded and repeated washing step twice. Diluted lysate supernatant were added into the equilibrated GFP-Trap[®]_A beads and sample was incubated for 1 hour at 4°C. Following that, beads were washed three times with 500 μ L of wash buffer (10mM Tris/Cl pH7.5, 150mM NaCl and 0.5mM EDTA) and centrifugation. Suitable volume of buffer was added to resuspend the beads and kept at 4 °C for future experiments. The resuspended beads were directly used for SETD3 *in vitro* methylation assay.

3. Results

3.1 Actin is the potential cytosolic substrate of SETD3

The first objective of this project is to determine whether SETD3 methylates any non-histone protein. For identification of potential substrates in different subcellular compartments, inactive mutant (MUT-) SETD3 was generated where the histidine 279 was mutated to arginine (Bunjamin et al, unpublished data). Following that, *in vitro* SETD3-methylation assay was conducted with BOSC23 cell lysate. As shown in Figure 3.1.1, SETD3 methylate non-histone proteins which are found at 37, 42 and 45 kDa (as indicated by red arrows). Methylation assays with fractionated BOSC23 cell lysate have conducted to examine the subcellular localization of the methylated proteins (data not shown). However, the result is not conclusive because the signal generated by MBP-SETD3 methylation become less evident in the cytosolic fraction. This could be due to high methyltransferase activity of endogenous SETD3 which is primarily found in the cytosol of BOSC23 cells. To address this issue, *in vitro* methylation assay with size exclusion fractionated lysates could be conducted. Size exclusion fractionation will separate endogenous SETD3 from the potential SETD3 substrates.

According to Figure 3.1.1, we speculated that actin to be one of the potential substrates, which has a molecular weight about 42 kDa. Subsequent *in vitro* SETD3 methylation assay was conducted in the presence of purified monomeric actin (G-actin) to determine whether the increased signal observed at size around 40- 45 kDa is indeed actin. As shown in Figure 3.1.2A, actin was methylated by WT-SETD3, but to a lesser extent by MUT-SETD3. In the biological system, actin can exist in two different forms, which are the monomeric and polymeric form. Thus, *in vitro* SETD3 methylation assay with polymerized actin (F-actin) was conducted to examine whether SETD3 methylation activity would be affected when actin is in filament structure. Based on the result (Figure 3.1.2B), SETD3 was able to methylate actin filaments. Native gel electrophoresis was conducted to validate the polymerization of actin, as shown in Figure 3.1.2C. In summary, SETD3 exerts methylation activity to both monomeric and polymeric forms of actin.

Figure 3.1. 1 SETD3 methylate proteins at the size around 37-45kDa

In vitro SETD3 methylation assay was conducted with total lysate from BOSC23 cells, wild type (WT) SETD3, Mutant (Mut) SETD3, and 100 nci of [methyl-3H]-S-Adenosyl]-L-Methionine ([3H]-SAM) (PerkinElmer) in methylation buffer (50 mM Tris [pH 8.5], 20mM KCl, 10mM MgCl₂, 10mM beta-mercaptoethanol, 250mM sucrose). Left panel shows the total protein included in the reaction through coomassie brilliant blue staining. Right panel shows the methylation signal generated from tritium labeled methyl group. Red arrow indicates the protein bands which show increased signal in the reaction with wild-type SETD3. The corresponding molecular weight of these protein is around 37, 42, and 45 kDa. This data set was generated in collaboration with Mr. Alex Wong Xin Fah.

Figure 3.1. 2 SETD3 methylates both monomeric and polymeric form of actin in vitro

[A] *In vitro* SETD3 methylation assay was conducted with purified G-actin. Left panel shows the total protein included in the reaction through coomassie brilliant blue staining. Right panel shows the methylation signal generated from tritium labeled methyl group. [B] *In vitro* SETD3 methylation assay was conducted with polymerized actin. Left panel shows the total protein included in the reaction through coomassie brilliant blue staining. Right panel shows the methylation signal generated from tritium labeled methyl group. [C] Native gel image containing G-actin (monomeric) and F-actin (polymeric). Red arrow indicates the position of F-actin in the native gel.

3.2 SETD3 is a Self-Methylating Lysine Methyltransferase

Methylation of methyltransferase is reported as one important feature that has regulatory effect (Chin 2007, Dillon 2013). To determine whether methylation of SETD3 occurs in *cis* or *trans*, *in vitro* methylation assay with SETD3-GFP, active and inactive MBP-SETD3 was performed (Figure 3.2.1). We found that active SETD3-GFP did not cross-methylate the inactive MBP-SETD3 (MUT MBP-SETD3), which indicates that the methylation of SETD3 might occur in *cis*.

In addition, methylation of SETD3 was also examined in the presence of its cytosolic and nuclear substrates. As shown in Figure 3.2.2A, SETD3 methylation signal remained constant despite the varying concentration of actin. On the contrary, the level of self-methylation of SETD3 was affected by its nuclear substrate. SETD3 methylation signal was observed to be decreased with the increase in amount of histone octamer (Figure 3.2.2B). Self-methylation and nuclear substrate methylation by SETD3 seems to be inversely related (Figure 3.2.2B).

Figure 3.2. 1 The methylation of SETD3 might occur in *cis*

Approximately 6×10^6 BOSC23 cells were transfected with pEGFP-SETD3 plasmids and followed by lysate extraction after 48 hours. GFP-pull down was then performed to obtain SETD3-GFP. *In vitro* methylation assay using MBP-SETD3 and SETD3-GFP was conducted. Left panel shows the total protein included in the reaction through coomassie brilliant blue staining. Right panel shows the methylation signal generated from tritium labeled methyl group.

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3.3 SETD3 Could Methylate Actin at Multiple Lysine Residues

Understanding the methylation site on actin is crucial for future functional studies. Two actin lysine methylation sites were identified so far, which are Lys-84 and Lys-326 (Vandekerckhove 1984, Li 2013). Methyltransferases responsible for methylation at Lys-84 and Lys-326 are still unknown. Actin mutants with amino acid substitutions at Lys-84, Lys-326 or Lys-328 (residue close to the identified methylation site) with alanine were generated and subsequently used in the *in vitro* SETD3 methylation assay. As shown in result, SETD3 methylated K84A actin mutant with the same intensity as wild-type actin, suggesting that actin Lys-84 is unlikely to be the methylation site for SETD3 (Figure 3.3.1A). Similarly, actin methylation by SETD3 was not disrupted by the mutation at Lys-326 and Lys-328. Hence, actin Lys-326 and Lys328 may not be the methylation sites for SETD3 (Figure 3.3.1B).

As shown previously, SETD3 methylated actin in both monomeric and filamentous state (Figure 3.1.2). Hence, the potential actin methylation site by SETD3 would be the lysine residues that are found on the surface of polymerized actin. Chemical methylation method was used to examine lysine reactivity on actin in the polymerized state (Lu 1981). Respective lysine residues Lys-50, Lys-118, Lys-191, Lys-237, Lys-290, Lys-335, and Lys 372 were found to be more reactive towards methylation in polymerized actin (Lu 1981). Site directed mutagenesis was conducted at these lysine residues followed by *in vitro* methylation assay. According to the result, SETD3 methylation activity is not completely hindered by the mutation. However, reduced methylation was observed in the mutation at Lys-290, Lys-335 and Lys-372 (Figure 3.3.1C, D). This implies that SETD3 could facilitate methylation at multiple lysine residues on actin.

Figure 3.3. 1 SETD3 could methylate actin at multiple Lysine residues

[A] Site directed mutagenesis was conducted to replace the respective lysine residues with alanine at the 3xFLAG-tagged actin and subsequently pulled down. *In vitro* SETD3 methylation assay were performed with 3xFLAG-tagged Lys-84 mutant actin (K84A) and 3xFLAG-tagged wild type actin. Red arrow indicates the position of 3xFLAG- actin at 50 kDa. Left panel shows the total protein included in the reaction through coomassie brilliant blue staining. Right panel shows the methylation signal generated from tritium labeled methyl group. [B] *In vitro* SETD3 methylation assay was performed with 3xFLAG-tagged Lys-326, Lys-328 mutant actin (K326A and K328A) and 3xFLAG-tagged wild type actin. [C] *In vitro* SETD3 methylation

assay was performed with 3xFLAG-tagged wild type actin, Lys-50 (K50A), Lys-118 (K118A), Lys-191 (K191A), Lys-237 (K237A), Lys-290 (K290A), Lys-335 (K335A) and Lys-372 (K372A) mutant actin. [D] Total protein normalization is adopted to quantify the methylation signal mediated by SETD3. The calculated value from mutant samples was compared with the value from WT sample to generate methylation intensity ratio (%). Experiment n=2. This experiment was conducted in collaboration with our attachment student, Mr. Yeo Kian Hua.

3.4 SETD3 Conditional Knockout Mouse Model

SETD3 conditional knockout mouse model (SETD3^{f/f}) was generated using CRISPR/Cas9 technology. Two loxP sites with exogenous NheI site were introduced into the SETD3 loci upstream of exon 3 and downstream of exon 4 (floxed SETD3 allele), as shown in figure 3.4.1A. Excision of exon 3 and 4 by Cre recombinase would result in a frameshift and introduce a premature stop codon. Two pairs of primers were designed to PCR genotype the mice containing both 5'loxP site and 3'loxP site. The floxed SETD3 allele (targeted) should have longer PCR products (~40bp) compared to the wild-type allele (Figure 3.4.1B/C). This mouse model with floxed SETD3 allele was first crossed to Mx1-cre transgenic mouse. Mx1 promoter is highly activated in response to interferon that is usually produced upon viral infection. Induced expression of Cre recombinase under the regulation of the Mx1 promoter could be achieved by administration of synthetic double stranded RNA [poly (I): poly(C)] (R. Kühn 1995). In this way, we are able to induce selective deletion of SETD3 in the interferon-responsive cells hematopoietic progenitors including T and B cell precursors, monocytes, macrophages, and neutrophils (Ichikawa 2004, Maltzman 2007, Yang 2013). Thereby, examination of SETD3's roles in different immune cells can be conducted simultaneously. Deletion efficiency of Mx-cre SETD3^{f/f} mice (MT) upon poly (I): Poly (C) injection was confirmed by PCR genotyping. The PCR genotyping was conducted with three primers namely 5VF1, 3VF1, and 3VR1. As shown in Figure 3.4.1D, PCR product at size around 362 bp was generated from 3' loxP site of the targeted allele. Additional PCR product at size around 278bp was generated from MT bone marrow and thymus, which indicates successful deletion of SETD3 allele (ko, for knockout).

Figure 3.4. 1 Generation of conditional SETD3 knockout mice

[A] Schematic representation of CRISPR/Cas9 based SETD3 targeting strategy. Single guide RNA (sgRNA) targeting SETD3 loci and a specific pair of oligodeoxynucleotides (ODNs) containing loxP site (green triangle) with an exogenous NheI site were introduced into the mouse genome by pronuclear injection. [B & C] PCR screening strategy (B) and expected PCR product size (C) from the targeted SETD3 loci. [D] PCR typing results of genomic DNA isolated from bone marrow, thymus cell and tail of SETD3^{f/f} (WT) Mx-cre SETD3^{f/f} mice (MT). This mouse model is generated in collaboration with Transgenic Mouse Model Core, National Taiwan University.

3.5 SETD3 Possibly Regulates Lymphocyte and Granulocyte Development

In the immune profiling of Mx-cre SETD3^{f/f} mice (MT), we examined the cells from bone marrow and thymus. Cells from spleen and lymph nodes were not examined due to low efficiency of SETD3 deletion, similarly to Mx-cre EZH2^{f/f} mice (Simon 2012). The knockout of SETD3 has caused modest abnormalities in the B cell development and neutrophils production. As shown in Figure 3.5.1 A/C, there was about 25% increase of SETD3-deficient pro-B cells (IgM⁻B220⁺CD43⁺) population compared to wild-type. There was no difference in the B220^{hi} IgM⁺ mature B cell and B220^{lo} IgM⁺ immature B cell populations. SETD3 knockout possibly causes suboptimal progression from pro-B to pre-B cells however with little effects in overall B cells generation. This phenomenon could arise from incomplete blockade of transition or supplementary mechanisms to support downstream B cell development. In addition, poly (I): poly(C) injected Mx-cre SETD3^{f/f} mice showed about 32% reduction of neutrophil (Ly6G^{hi}Ly6C⁺) population in the bone marrow, whereas the other bone marrow myeloid cell populations remained constant. Bone marrow neutrophil production might be regulated by SETD3 (Figure 3.5.1 B/D). The differences observed in B cell and neutrophils are modest, thus more experiments need to be conducted to examine if the differences are statistically significant. Activation of SETD3-deficient bone marrow derived dendritic cells (BMDC) and macrophages (BMDM) were also examined. Complete SETD3 deletion in BMDCs and BMDMs was confirmed by western blot analysis (Figure 3.5.2 C). *In vitro* stimulation of BMDCs and BMDMs were not affected in the absence of SETD3 (Figure 3.5.2A/B). Thus, SETD3 appears to be dispensable for the activation of BMDC and BMDM.

The distribution of thymocytes is severely affected by the knockout of SETD3. The frequency of double positive thymocytes (DP), CD8 single positive thymocytes (SP), and CD4 single positive thymocytes (SP) were remarkably perturbed in poly (I): poly(C) injected Mx-cre SETD3^{f/f} mice (Figure 3.5.3A). In addition, the CD4⁻CD8⁻ double negative thymocytes (DN) seem to be affected in the absence of SETD3. The frequency of SETD3-deficient DN1 (CD44⁺ CD25⁻), DN2 (CD44⁺ CD25⁺), DN3 (CD44⁻ CD25⁺) thymocytes were much lower compared to that in WT mice. In contrast, SETD3-deficient

DN4 (CD44⁺ CD25⁻) population was accumulated (Figure 3.5.3B). This could indicate that the development of thymocytes was blocked at the DN4 to DP transitions in the absence of SETD3. Usually DN4 thymocytes should express intracellular TCR β (icTCR β) chain upon completion of the TCR β chain VDJ recombination at DN3 stage. To better determine the underlying molecular mechanisms for this developmental block, we will include intracellular TCR β (icTCR β) staining in our future experiments (Mombaerts 1992).

Figure 3.5. 1 SETD3 could have regulatory effect on B cell development and neutrophil production

[A] Bone marrow cells were obtained from SETD3^{f/f} (WT) and Mx-cre SETD3^{f/f} (MT) mice 8 weeks upon Poly(I)Poly(C) injection and analysed by flow cytometry. Left panel shows population from B220^{hi} IgM⁺ mature B cell (WT:3.56%, MT:4.18%), B220^{lo} IgM⁺(immature B cell WT:2.08%, MT:2.29%) and IgM⁻ population(WT:89.6%, MT:89.7%). IgM⁻ populations were further analyzed as shown in right panel, including CD43⁺ Pro B cells (WT: 2.66%. MT: 4.50%) and CD43⁻ Pre B cells (WT: 6.90%, MT: 7.32%). [B] Left panel shows population from Ly6G^{hi} Ly6C⁺ Neutrophils (WT: 40.6%, MT: 34.0%) and Ly6G⁻ constitute of monocytes and macrophages. Right panel shows the F4/80⁺ CD11b⁺ macrophage population (WT: 17.5%, MT: 16.1%). [C] Absolute cell number of mature B cell, immature B cell, pro-B cell and pre-B cell were

calculated and represented in the histogram. [D] Absolute cell numbers of macrophage, neutrophil and Ly6G⁺ population were calculated and represented in the histogram. These data sets were generated in collaboration with Ms. Maegan Bunjamin and Mr. Lim Jun Feng.

Figure 3.5. 2 SETD3 does not regulate the activation processes of bone marrow derived dendritic cells (BMDC) and macrophages (BMDM)

[A] Bone marrow cells were obtained from SETD3^{fl/f} (WT) and Mx-cre SETD3^{fl/f} (MT) mice. The bone marrow cells were cultured in complete RPMI medium containing 100x dilution of GM-CSF supernatant. At day 7, BMDCs were stimulated with 100ng/ml LPS overnight. After LPS stimulation, both unstimulated and LPS stimulated BMDCs were collected for flow cytometry analysis. The dot plot shows population of immature dendritic cells (CD11c⁺ MHCII^{lo}) and activated dendritic cells (CD11c⁺ MHCII^{hi}). [B] The bone marrow cells were cultured in DMEM medium containing 20% FCS, 1% Pen/Strep, 1% L-Glutamine, 500ul Sodium Pyruvate, 10% L929 conditioned media. At day 6, BMDMs were stimulated with 100ng/ml LPS. After overnight LPS stimulation, both unstimulated and LPS stimulated mature BMDMs were obtained for flow cytometry analysis. The middle panel shows the population of macrophages (F4/80⁺ CD11b⁺). Activated macrophages showed higher expression of CD69 (right panel). [C] Examination of SETD3 protein expression in BMDC and BMDM from SETD3^{fl/f} (WT) and Mx-cre SETD3^{fl/f} (MT). These data sets were generated in collaboration with Ms. Maegan Bunjamin and Mr. Lim Jun Feng.

Figure 3.5. 3 SETD3 could have regulatory role in T cell development

[A] Thymus cells were obtained from poly(I)poly(C) treated *SETD3^{fl/fl}* (WT) and *Mx-cre SETD3^{fl/fl}* (MT) mice followed by flow cytometry analysis. The dot plots show the CD4⁻ CD8⁻ double negative T cells (DN), CD4⁺ CD8⁻ single positive T cells (CD4 T cells), CD4⁻ CD8⁺ single positive T cells (CD8 T cells), and CD4⁺ CD8⁺ double positive T cells (DP). [B] The dot plots show the development of double negative T cells (DN) population including DN1 (CD44⁺ CD25⁺), DN2 (CD44⁺ CD25⁻), DN3 (CD44⁻ CD25⁺), and DN4 (CD44⁻ CD25⁻). [C] The dot plots show the activated T cells in both *SETD3^{fl/fl}* (WT) and *Mx-cre SETD3^{fl/fl}* (MT) mice. Activated T cells have higher expression of CD69. These data sets were generated in collaboration with Ms. Maegan Bunjamin and Mr. Lim Jun Feng.

3.6 SETD3 Expression Increased in B Cells upon Activation

One of our objectives is to examine the potential function of SETD3 in the cytosol of immune cells. In this experiment, B cells were isolated from spleen through MACS® column by depletion using CD43 beads, and stimulated *in vitro* followed by the protein level check in unstimulated and activated B cells. Isolated resting B cells can be activated in the presence of stimulants, the most common being the IgM (Fab)₂ (T-independent stimulation), anti-CD40 antibody (T-dependent activation), and LPS (T-independent stimulation) (Wortis 1995, Hua 2013). According to Figure 3.6.1 A/C, B cells expressed

increased level of SETD3 in the cytosolic compartments upon stimulation by both T-dependent and T-independent signals. Expression of CD86 was measured to determine the activation status of B cell, as shown in Figure 3.6.1 B/D. Based on the results, we interpret that SETD3 may have a functional role in the activation process or effector function of activated B cells. In previous result, actin was identified as the potential cytosolic substrate of SETD3 (Figure3.1.2). We speculate that increased expression of cytosolic SETD3 in activated B cells may result in hyper methylation of actin which could potentially contribute to the development and function of lymphocytes. Hence, further experiments are required, such as to examine peripheral B cell in SETD3 knockout mouse model in combination with germinal center specific transgenic mice (AID-cre or IgG1-cre), methylation level on actin *in vivo* and biochemical functional assays (Casola 2006, Robbiani 2008).

Figure 3.6. 1 B cells upregulated SETD3 expression upon stimulation

[A] SETD3 expression level in unstimulated and stimulated (by IgM F(ab)2 or Anti-CD40 or both) B cells. GAPDH level serves as a loading control. [B] Flow cytometry analysis of CD86 expression in unstimulated and stimulated B cell. Activated B cells express higher level of CD86 molecules. [C] SETD3 expression level in unstimulated and LPS stimulated B cells. GAPDH level serves as a loading control. [D] Flow cytometry analysis of CD86 expression in unstimulated and LPS stimulated B cells. Activated B cells express higher number of CD86 molecules.

4. Discussion

4.1 Self Methylation of SETD3

In this research project, we determined that SETD3 is methylated *in vitro* (Figure 3.2.1). However, due to some technical shortcomings and limited number of replications, some results are inconclusive. It is still unclear whether the methylation of SETD3 occurs in *cis* or *trans*, since low concentration of active SETD3-GFP was used in the experiment shown here. To address the pitfall, we could include the inactive SETD3-GFP in future experiments to examine whether active MBP-SETD3 (in high concentration) would cross-methylate the inactive SETD3-GFP. The preliminary result also revealed that methylation on SETD3 is preferred when low concentration of histone octamer was presented (Figure 3.2.2B). This observation seems to be similar to the PRMT8 self-methylation model. The lack of unmethylated substrate causes self-methylation of PRMT8 leading to down-regulation of PRMT8 activity (Dillon 2013). However, we discovered that actin does not interfere with methylation on SETD3 (Figure 3.2.2A). Based on our data, we propose a dual-pocket-binding model for SETD3's enzymatic function in which actin might interact with an alternative binding site as histone. The dual-pocket-binding model is established along with the discovery of self-methylation on MLL1. Self-methylation of MLL1 is found to be inhibited only by unmethylated but not tri-methylated histones (H3Lys-4 and H3Lys-9), which suggests the existence of different binding sites on MLL1 for unmodified and tri-methylated histones and only the former binding sites compete with MLL1 self-methylation (Patel 2014). *In vitro* methylation assay with pre-methylated SETD3 and crystallography experiment may help to justify the hypothesis, which is further discussed in section 6.

4.2 Posttranslational Modifications (PTMs) on Actin

One of the main objectives of this study is to identify cytosolic substrates of SETD3. Here, we identified actin as a potent substrate of SETD3. Actin is known to be instrumental in numerous cellular behaviors through protein-protein interactions. The ability to switch between monomeric (G-actin) and filamentous (F-actin) forms illustrates the dynamics of

actin and make it a vital protein in various cellular functions, ranging from regulation of transcription to cell motility and cell shape maintenance (Reviewed in Dominguez 2011).

Dynamic activities of actin are well regulated by PTMs (Reviewed in Pollard 2009). Along with methylation, multiple PTMs on actin are reported including acetylation, phosphorylation, glycosylation, and more (Reviewed in Terman 2013). Acetylation of actin commonly occurs at the N-terminal end. Study has reported that such acetylation reinforce the interaction between actin and myosin (Abe 2000). In addition, actin is also acetylated at Lysine-61 and that stabilizes the stress fiber formation (Kim 2006). Glycosylation is found at Serine-54 of cardiac actin that is important for the regulation of myofilament Ca^{2+} activation (Ramirez-Correa 2008). Phosphorylation of actin regulates the activation of epithelial sodium channel mediated by cAMP-dependent protein kinase (PKA) (Prat 1993).

Study has unveiled the regulatory effect of methylation on actin function. Lys-84 demethylation by ALKBH4 allows actin binding to non-muscle myosin II which is important to support cytokinesis and cell migration through the maintenance of actomyosin dynamics (Li 2013). Collectively, our result showed that SETD3 did not catalyze methylation at actin Lys-84. In addition, our preliminary experiment showed that SETD3 methylated F-actin and 9 lysine residues were predicted as potential methylation site due to their high reactivity towards chemical methylation even in the polymeric form of actin (Lu 1981). We found that actin with single mutation at Lys-290, Lys-335 and Lys-372 exhibited reduction in the methylation mediated by SETD3 (Figure 3.3.1D). This indicates that Lys-290, Lys-335 and Lys-372 could be potential methylation sites. However, question arose whether F-actin structure was disrupted during the *in vitro* methylation assay (Figure 3.1.2B). Therefore, additional *in vitro* SETD3 methylation assay was conducted with the phalloidin which is used to stabilize F-actin structure throughout the reaction. Surprisingly, SETD3 did not methylate actin in the phalloidin-actin complex (data not shown). This result remains inconclusive because phalloidin binds to actin near the predicted methylation site Lys-290, Lys-335 and Lys-372, thus it could possibly block SETD3-mediated actin methylation (Faulstich 1993). To address this question, future methylation assays could be

conducted with the depolymerization inhibitors, such as dolastatin 11 and hecotochlorin that bind to actin at different site from the phalloidin binding site (Marquez 2002, Oda 2003).

Overall, multiple PTMs have been identified as the “control switch” regulating actin-related events. The discovery of actin being the potential substrate for SETD3-mediated methylation may contribute to interesting functional roles of SETD3 particularly in cytoskeleton related events. However, further experiments addressing the effect of actin methylation in the dynamics of actin polymerization/depolymerization or the interaction of methylated actin with other actin regulatory proteins are required to determine the physiological role of SETD3-mediated actin methylation.

4.3 Actin Cytoskeleton Events in Hematopoietic Cells

In this study, we are interested in investigating the physiological functions of SETD3 in immunological system. During B cell activation, cytoskeleton reorganization is crucial for the signal transduction of B cell receptor (BCR) by controlling the organization of signaling molecules and receptors (Song 2014). Pro-to-Pre B cell development may be mildly affected when SETD3 is knockout. In addition, SETD3 is highly upregulated in the germinal center B cells (Figure 1.10.2) and we also observed an upregulation of SETD3 protein in the cytosol of activated B cells, it is possible that SETD3 plays a critical role for B cells during germinal center reaction. It is worthwhile to investigate the function of SETD3 in regulating B cell development and activation. We could first identify SETD3 subcellular localization in the B cell precursors and mature B cells. Following that, SETD3 mediated methylation at histone and actin could be examined in SETD3 deficient B cells.

In this project, we observed visible changes in T cell development when SETD3 is eliminated. We postulate that SETD3 may regulate T cell development beyond DN3 resulting in a severe developmental block during the transition of DN4 to DP stage. However, cytoskeleton remodeling for early T cell development have not been reported. The localization of SETD3 and dynamics of actin assembly could be examined in the SETD3-deficient T cell precursors. In addition, the roles of actin have mostly discussed in the aspect of T cell effector functions, for instance activation of T-cell, formation of

immunological synapse (IS) and signaling (Wülfing 1998). In future, experiments including activation and effector functional test of SETD3-deficient T cells could be carried out to determine SETD3 regulatory potential of the reported function. To generate SETD3-deficient T cells, we can utilize CD4-cre or distal Lck-cre transgenic mice (Lee 2001).

In SETD3-deficient mice, we observed moderate differences in the neutrophil population as compared to WT mice (Figure 3.5.1B/D). Based on the current data, we deduced that SETD3 may have regulatory potential in the neutrophil production. However, it remains unknown whether cytoplasmic or nuclear SETD3 activity is affecting the neutrophils production. Thus, further experiments are required to determine if the observed differences are statistically significant and to examine subcellular localization and functional aspects of SETD3 in neutrophils. Neutrophils being the motile cell type heavily rely on cytoskeletal dynamics to regulate the motion processes (Taylor 2014). On top of that, early study has shown that neutrophil degranulation requires proper organization of actin as well (Boyles 1981). Therefore, it will be interesting to find out the role of SETD3-mediated actin methylation in neutrophils effector functions.

The absence of SETD3 did not impair the development and activation of macrophages and dendritic cells. However, we discovered that SETD3 appears to be localized in the cytoplasmic compartment of macrophages (Bunjamin et al, unpublished data). Study has reported the prominent role of actin dynamics in macrophage effector functions, which includes target sensing, formation of different membrane structures, and phagocytosis (Reviewed in Freeman 2014). Hence, it is plausible that SETD3-mediated actin methylation regulates the effector function of macrophages. In addition, actin-controlled migration governs the chemokine induced migration for mature dendritic cells (Garrett 2000, Vargas 2016). To determine if SETD3-mediated actin methylation is involved in the reported function, we could examine the subcellular localization of SETD3 in mature dendritic cells followed by functional examination.

5. Conclusion

SETD3 is a protein methyltransferase that may be required for proper functioning of various tissues and hematopoietic lineage cells. Recent publications have shown that SETD3 has function in regulating muscle differentiation as well as involved in oncogenesis (Eom 2011, Chen 2012). However, functional implications of SETD3 self-methylation or cytosolic functions of SETD3 has not been reported. The main aim of our study was to characterize SETD3's enzymatic activity and physiological function. Four major findings from this study are (i) the identification of a novel cytosolic substrate (ii) characterization of SETD3 self-methylation activity (iii) perturbed development of T cell, B cell and neutrophils in the absence of SETD3 (iv) upregulation of SETD3 expression in activated B cells. Based on these findings, SETD3 possibly has functional roles beyond nucleus in the development as well as effector functions of immune cells. We will conduct experiments designed to elucidate the functional roles of SETD3 in the nucleus as well as in the cytosol of immune cells. This certainly will provide novel insights of the regulatory effect of protein methylation on the development and functions of immune cells.

6. Future Direction

Epigenetic regulation of gene expression is important in physiological and pathological conditions through the modifications of histone proteins (Reviewed in Kouzarides 2007). Overexpression of SETD3 induces transcription of muscle-related genes, such as myogenic factor (Myf6), myogenin, and muscle creatine kinase (MCK), which stimulate muscle cell differentiation (Eom 2011). A truncated SETD3 without SET domain, which is resulted from translocation of Chr12 into the Ig λ locus on Chr16, is proven to be oncogenic (Chen 2012). However, the function of SETD3 in cytosol remains to be elucidated.

Our data reveal actin is the potent substrate of SETD3 in cytosol. Additionally, our preliminary results have shown that T cell, B cell and neutrophil development may be perturbed in the absence of SETD3. Other than that, our result also shows the upregulation of cytosolic expression in B cell upon stimulation. In this project, we will further characterize enzymatic activity of SETD3, determine the functional role of SETD3-mediated methylation of actin and the involvement of SETD3 in the development of immune cells as well as in the effector functions of activated B cells.

Aim1: To characterize the methylation activity of SETD3

Preliminary data showed that SETD3 methylated actin *in vitro*. Identification of the SETD3-mediated actin methylation site will be conducted through mass spectrometry and site-directed mutagenesis. Determining the methylation site at actin is essential to generate specific antibody for future functional assays. Therefore, we could examine SETD3 mediated methylation at actin *in vivo* to determine the cause of the phenotype observed in Mx-cre SETD3^{f/f} mice. SETD3 exhibits self-methylation activity and the activity differ in the reaction with its cytosolic or nuclear substrate. We speculate that histone shares the same binding site with self-methylation. *In vitro* methylation assay can be conducted with pre-methylated SETD3, histone and cytosolic substrates. If SETD3 follows the dual-pocket-binding model, pre-methylated SETD3 will probably methylate actin but not histone. Future crystallography experiments can be conducted to visualize the interaction of SETD3 with its cytosolic and nuclear substrates. Similarly, SETD3 self-methylation site can be identified through mass spectrometry and site-directed mutagenesis. Generation of methyl-

SETD3 specific antibodies will permit further characterization of the physiological implications of SETD3 self-methylation.

Aim 2: To determine additional cytosolic substrates of SETD3

Study has reported that SETD3 interacts with 174 proteins found in the cytosolic and nuclear compartments including actin and actin binding protein LIM protein 1 (ABLIM1) (Cohn 2016). Thus, we should first examine SETD3 methylation activity on ABLIM1 and other actin-related proteins. Simultaneously, methylation profiling can be conducted through peptide microarray or the proteome-wide enrichment of methylated protein method as described by Carlson (2014). Subsequently, functional assays will be conducted to elucidate SETD3 regulatory role on the newly identified substrates in various immune cells as described in the next aim.

Aim3: To determine whether SETD3-mediated histone methylation or actin methylation is involved in the development, activation and effector functions in various immune cells.

SETD3 potentially regulates the development of neutrophils, B-cell and T- cell precursors. It remains unknown whether SETD3 acts in cytosol or nucleus. Microscopic imaging can be conducted on these immune cell precursors to determine the subcellular localization of SETD3 during developmental stage. If SETD3 is mainly found in the cytosolic compartment, subsequent biochemical assays can be conducted to determine level of SETD3 mediated actin methylation in WT and SETD3-deficient neutrophils, B-cell and T-cell precursors. However, if majority of SETD3 are found in the nucleus, we could perform transcriptome analysis to identify downstream SETD3-regulated gene. In addition, SETD3 expression is upregulated upon stimulation of B-cells and thus it may be involved in the activation or effector functions. We could immunize WT and AID-cre SETD3^{fl/fl} mice to examine whether activation of germinal center B cells is affected *in vivo* (Robbiani 2008). Study has reported the importance of actin dynamics in signal transduction during activation (Song 2014). Live imaging of the SETD3-deficient B cell during stimulation would help determine whether actin reorganization is disrupted. We will also further investigate the role of SETD3 in the mature T cells, neutrophils, macrophages and dendritic

cells. To study the effects of SETD3-mediated methylation in regulating various immune cells *in vivo*, we will utilize several cell type specific Cre transgenic mice, such as CD4-cre or Lck-cre for T- cell, Lyz2-cre for myeloid cells (granulocytes and mature macrophages) and CD11c-cre for dendritic cells (Clausen 1999, Lee 2001, Caton 2007).

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