Investigating the Role of mTOR in Histone Methylation

by

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ABSTRACT

mTOR coordinates a variety of signals, including nutrient and oxygen availability, growth factors, and hormones, to stimulate cell growth and proliferation. This pathway is also frequently hyperactivated in neoplasia. Changes in gene expression, including those caused by epigenetic dysregulation, represent a hallmark of cancer. However, the role of mTOR in epigenetic reprogramming in the context of neoplasia remains largely unknown. In this study, we investigated the impact of modulation of mTOR signaling on histone methylation. We observed that constitutive activation of mTORC1 signaling caused by the loss of TSC2 is paralleled by the induction of H3K27me3, a histone methylation mark associated with gene repression. This was mediated via the 4E-BP1-EZH2 axis. Surprisingly, mTOR inhibition also induced an increase in H3K27me3 levels. Further investigation into the mechanism behind H3K27me3 induction following mTOR inhibition revealed that it appears to be independent of TSC2 status in the cell, cell cycle progression and H3K27me3 demethylation. Our findings also suggest that the level of the H3K27 writers, EZH1/2, could not fully explain H3K27me3 induction upon mTOR inhibition. Using a model of H3K27M mutant cells, we provide evidence that PRC2 may contribute to the induction of H3K27me3 following mTOR inhibition. Furthermore, cells with low H3K27me3 levels displayed reduced susceptibility to mTOR inhibition, indicating that H3K27me3 induction may play a partial role in mediating the anti-proliferative effects of mTOR inhibitors. Overall, our findings suggest that mTOR dysregulation triggers H3K27me3 induction, and H3K27me3 induction upon mTOR inhibition may contribute to some extent, to anti-proliferative effects of mTOR inhibition. This discovery lays the groundwork for further research to establish the role of mTOR in epigenetic reprogramming in the context of neoplasia.

ABRÉGÉ

La voie de mTOR coordonne une variété de signaux, y compris la disponibilité des nutriments, de l'oxygène, des facteurs de croissance, et des hormones. Cela afin de stimuler la croissance et la prolifération cellulaire. Cette voie est également fréquemment hyperactivée dans la néoplasie. Les changements dans l'expression génétique, y compris ceux causés par un dérèglement épigénétique, sont des caractéristiques du cancer. Cependant, le rôle de mTOR sur la reprogrammation épigénétique dans le contexte de la néoplasie reste largement inconnu. Dans cette étude, nous avons investigué l'impact de la modulation de la signalisation de mTOR sur la méthylation des histones. Nous avons observé que l'activation constitutive de la signalisation de mTORC1, causée par la perte de TSC2, est accompagnée par l'induction de H3K27me3, une marque de méthylation des histones associée à la répression génétique. Celle-ci était médiée par l'axe 4E-BP1-EZH2. Étonnamment, l'inhibition de mTOR a également entraîné une augmentation des niveaux de H3K27me3. Des investigations supplémentaires sur le mécanisme de l'induction de H3K27me3 après l'inhibition de mTOR ont révélé qu'elle semble être indépendante de l'état de TSC2 dans la cellule, de la progression du cycle cellulaire, et de la déméthylation de H3K27me3. Nos résultats suggèrent également que le niveau des enzymes EZH1/2 qui écrivent H3K27 ne pourrait pas expliquer entièrement l'induction de H3K27me3 lors de l'inhibition de mTOR. En utilisant un modèle de cellules mutantes H3K27M, nous fournissons des preuves que le complexe PRC2 pourrait contribuer à l'induction de H3K27me3 après l'inhibition de mTOR. De plus, les cellules présentant des niveaux bas de H3K27me3 ont montré une susceptibilité réduite à l'inhibition de mTOR. Ce qui indique que l'induction de H3K27me3 peut jouer partiellement un rôle dans la médiation des effets antiprolifératifs des inhibiteurs de

mTOR. Dans l'ensemble, nos résultats suggèrent que la dysrégulation de mTOR déclenche l'induction de H3K27me3 et que l'induction de H3K27me3 lors de l'inhibition de mTOR peut contribuer, dans une certaine mesure, aux effets antiprolifératifs de l'inhibition de mTOR. Cette découverte pave la voie pour établir le rôle de mTOR sur la reprogrammation épigénétique dans la néoplasie.

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"It is God Who arms me with strength and keeps my way secure" (Psalms 18:32).

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This work was performed at McGill University that is on land which has long served as a site of meeting and exchange amongst Indigenous peoples, including the Haudenosaunee and Anishinabeg nations. We acknowledge and thank the diverse Indigenous peoples whose presence marks this territory on which peoples of the world now gather.

PUBLICATION ARISING FROM THIS WORK

Chapter 3 is in preparation for submission:

HaEun Kim, Benjamin Lebeau, David Papadopoli, Predrag Jovanovic, Mariana Russo, Daina Avizonis, Masahiro Morita, Josie Ursini-Siegel, Lynne-Marie Postovit, Michael Witcher, and Ivan Topisirovic (2023) mTOR selectively modulates histone methylation which underpins its effects on proliferation. Manuscript in preparation for submission.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Aim 1. Investigated the impact of mTORC1 hyperactivation on histone methylation using TSC2-null MEFs, which possess constitutively active mTORC1 (Results presented in section 3.1).

- Observed that mTORC1 hyperactivation caused by TSC2 KO selectively alters the histone methylation mark H3K27me3.
- Confirmed that H3K27me3 alteration in mTORC1 hyperactivation is mediated through the 4E-BP1-EZH2 axis.

Aim 2: Examined the impact of mTOR inhibition on histone methylation and investigated the underlying mechanisms involved in regulating histone methylation upon mTOR inhibition (Results presented in sections 3.2-3.5).

- Observed that mTOR inhibition induces H3K27me3 independently of TSC2 status.

- Identified that the hypermethylation of H3K27 in response to mTOR inhibition is attributed to the involvement of PRC2 and not to cell cycle arrest induced by mTOR inhibition or caused by differential demethylation of H3K27me3.
- Demonstrated that PRC2 activity is essential for the induction of H3K27me3 upon mTOR inhibition, yet this effect does not involve changes in substrate levels, PRC2 assembly, or EZH2 localization, indicating the necessity for further investigation.

Aim 3: Determined the role of histone methylation in response to mTOR inhibition (Results presented in section 3.6).

- Confirmed that mTOR inhibition and H3K27me3 dysregulation are involved in reducing cell proliferation.
- Discovered that the effect of mTOR inhibition on cell proliferation may be partially mediated by the induction of H3K27me3.

PREFACE

This thesis is written in the traditional format. It is divided into five chapters as follows:

Chapter 1: Literature Review

Chapter 2: Experimental Procedures

Chapter 3: Results

Chapter 4: Discussion and Conclusion

Chapter 5: Bibliography

CONTRIBUTIONS OF AUTHORS

Chapter 1:

HaEun Kim wrote the review paper in collaboration with Dr. David Papadopoli.

Chapter 3:

HaEun Kim designed and performed the experiments, as well as generated, analyzed, and performed statistical analysis for the figures. Dr. David Papadopoli assisted with metabolomics (GC-MS and LC-MS). Dr. Ivan Topisirovic supervised the project. Dr. Michael Witcher and Dr. Lynne-Marie Postovit also provided project supervision. Dr. Benjamin Lebeau analyzed the RNA and ChIP-sequencing data.

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LIST OF ABBREVIATIONS

2'-O-Me: 2'-O-methylation

4-OHT: 4-hydroxytamoxifen

4E-BP: Eukaryotic initiation factor 4E-binding protein

AEBP2: Adipocyte enhancer-binding protein

ALKBH5: AlkB homolog 5

AMBRA1: Autophagy/beclin 1 regulator 1

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

asTORi: Active-site mTOR inhibitor

ATP: Adenosine triphosphate

BAD: BCL2 associated agonist of cell death

BIM: Bcl-2 Interacting Mediator of cell death

CAC: Citric acid cycle

CAD: Carbamoyl-phosphate synthetase

CATACOM: Catalytic antagonist of polycomb

CDK1/2: Cyclin-dependent kinase 1 or 2

CK1/2: Casein kinases 1 or 2

DEPTOR: DEP domain-containing mTOR-interacting protein

DIPG: Diffuse intrinsic pontine glioma

DMEM: Dulbecco’s modified eagle medium

DMKG: Dimethyl α-ketoglutarate

DMSO: Dimethyl sulfoxide

DNMT: DNA methyltransferase

DZNep: Deazaneplanocin A

EED: Embryonic ectoderm development

eEF2K: Elongation factor eEF2 kinase

eIF4A: Eukaryotic translation initiation factor 4A

eIF4B: Eukaryotic translation initiation factor 4B

eIF4E: Eukaryotic initiation factor 4E

Eif4ebp: Eukaryotic translation initiation factor 4E binding protein

EMT: Epithelial-to-mesenchymal transition

EPOP: Elongin BC and polycomb repressive complex 2-associated protein

ESI: Electrospray ionization

EV: Empty vector

EZH1: Enhancer of zeste homolog 1

EZH2: Enhancer of zeste homolog 2

FBS: Fetal bovine serum

FH: Fumarate hydratase

FRB: FKBP12-rapamycin binding domain

FTO: Fat mass and obesity-associated protein

GAP: GTPase-activating protein

GC-MS: Gas chromatography–mass spectrometry

GOF: Gain-of-function

H2AK119ub1: Mono-ubiquitylates histone H2A at lysine 119

H3K27ac: H3K27 acetylation

H3K27me1: Monomethylate histone H3 at lysine 27

H3K27me2: Dimethylate histone H3 at lysine 27

H3K27me3: Trimethylate histone H3 at lysine 27

H3K4me3: Trimethylate histone H3 at lysine 4

H3K9me2: Dimethylate histone H3 at lysine 9

H3K9me3: Trimethylate histone H3 at lysine 9

HAT: Histone acetyltransferase

HDAC: Histone deacetylase

HDB: HeLa dialysis buffer

HDM: Histone demethylase

HMT: Histone methyltransferase

HOTAIR: HOX transcript antisense RNA

IF: Immunofluorescence

IGF-1: Insulin-like growth factor 1

iRapKO: Inducible knockout MEFs deficient in the mTORC1-specific component Raptor

iRicKO: Inducible knockout MEFs deficient in the mTORC2-specific component Rictor

ISR: Integrated stress response

JARID2: Jumonji- and AT-rich interaction domain containing 2

JmjC: Jumonji C

JMJD3: Jumonji domain-containing protein 3

K27M: Lysine-27-to-methionine substitution

KDM: Histone lysine demethylase

LC-MS: Liquid chromatography–mass spectrometry

LKB1: Liver kinase B1

lncRNA: Long non-coding RNA

m1A: N1-methyladenosine

m5C: 5-methylcytosine

m6A: N6-methyladenosine

MEF: Mouse embryonic fibroblast

MFI: Mean fluorescence intensity

miRNA: MicroRNA

MMTV-PyMT: Mouse mammary tumour virus-polyoma middle tumour-antigen

MNase: Micrococcal nuclease

MRM: Multiple reaction monitoring

mRNA: Messenger RNA

MTBSTFA: N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide

MTF2: Metal response element binding transcription factor 2

mTOR: Mammalian/mechanistic target of rapamycin

mTORC1: mTOR complex 1

mTORC2: mTOR complex 2

mTORi: mTOR inhibitor

ncRNA: Non-coding RNA

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate-buffered saline

PcG: Polycomb group

PCL: Polycomb-like protein

PDK1: Phosphoinositide-dependent kinase 1

PFK: Phosphofructokinase

PGL: Paraganglioma

PHF1: PHD finger protein 1

PHF19: PHD finger protein 19

PI3K: Phosphoinositide 3-kinase

PIKK: Phosphatidylinositol 3-kinase-related kinase

piRNA: Piwi-interacting RNA

PLK1: Polo-like-kinase 1

Pol I: RNA polymerase I

PPARγ: Peroxisome proliferator-activated receptor gamma

PPP: Pentose phosphate pathway

PRC2: Polycomb Repressive Complex 2

PTEN: Phosphatase and tensin homolog

PTM: Post-translational modification

R-2HG: R-2-hydroxyglutarate

RAPTOR: Regulatory-associated protein of mTOR

RB: Retinoblastoma protein

RBBP4: Retinoblastoma-binding protein 4 or 7

REDD1: Regulated in development and DNA damage responses 1

RHEB: Ras homologue enriched in brain

RICTOR: Rapamycin-insensitive companion of mTOR

RPMI: Roswell Park Memorial Institute medium

rpS6: Ribosomal protein S6

RTK: Tyrosine kinase receptor

S-2HG: S-2-hydroxyglutarate

S6K: S6 kinase

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SD: Standard deviation

SDH: Succinate dehydrogenase

SEM: Standard error of the mean

SGK1: Serum- and glucocorticoid-regulated kinase 1

shRNA: Short hairpin RNA

siRNA: Short interfering RNA

SKAR: S6K1 Aly/REF-like substrate

SREBP: Sterol regulatory element-binding protein

SUMO: Small ubiquitin-like modifier

SUZ12: Suppressor of zeste 12 homolog

TFEB: Transcription factor EB

TIF-1A: Transcription initiation factor 1A

TSC: Tuberous sclerosis complex

TSC1: Tuberous sclerosis complex 1

TSC2: Tuberous sclerosis complex 2

TSS: Transcriptional start site

ULK1: Unc-51-like autophagy activating kinase 1

UTX: Ubiquitously transcribed tetratricopeptide repeat X

WT: Wild-type

α-KG: α-ketoglutarate

CHAPTER 1. Literature review

1. Mammalian/mechanistic Target of Rapamycin (mTOR)

mTOR is a protein kinase that plays a crucial role in regulating cell growth and metabolism in response to changes in nutrient availability, energy status, and other signals (Saxton & Sabatini, 2017; Zou et al., 2020). It is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK family) (Keith & Schreiber, 1995) and is conserved across different species, from yeast to mammals (Tatebe & Shiozaki, 2017). The mTOR pathway is regulated by a number of upstream kinases and signaling molecules, including the PI3K/AKT and the AMPK pathway (Cantley, 2002; González et al., 2020; Vanhaesebroeck et al., 2010). In response to various signals, these upstream pathways modulate mTOR activity, which in turn regulates downstream targets such as ribosomal protein S6 kinase (S6K), eukaryotic initiation factor 4E-binding protein (4E-BP), and AKT hereby influencing cell proliferation, growth and survival (Sabatini, 2017). In mammals, there are two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). While both complexes contain the mTOR at their core, they are composed of other complex-specific proteins (Papadopoli et al., 2019). mTORC1 is composed of mTOR, regulatory-associated protein of mTOR (RAPTOR), mLST8/GβL, PRAS40, and DEP domain-containing mTOR-interacting protein (DEPTOR) (Saxton & Sabatini, 2017). In contrast to mTORC1, mTORC2 is composed of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), and various other subunits such as DEPTOR, mSIN1 and Protor1/2 (Saxton & Sabatini, 2017) (Fig 1.1). Additionally, while mTORC1 regulates protein synthesis and autophagy in response to nutrient availability and stress signals, mTORC2 is involved in regulating multiple aspects of cell signaling, including survival, cytoskeletal dynamics, lipid homeostasis, and glucose metabolism.

Dysregulation of mTOR signaling has been implicated in a wide range of diseases, including cancer, metabolic disorders, and neurological disorders, making it an attractive target for drug development (Ciuffreda et al., 2010; Forbes et al., 2011; Inoki et al., 2005; Liu et al., 2009; Ma et al., 2010; Sawicka & Zukin, 2012; Zou et al., 2020). In fact, a class of drugs known as mTOR inhibitors, such as rapamycin and Ink128, is currently in the midst of clinical evaluation to treat some types of cancer and prevent organ rejection in transplant patients (Ali et al., 2022; de Fijter, 2017).

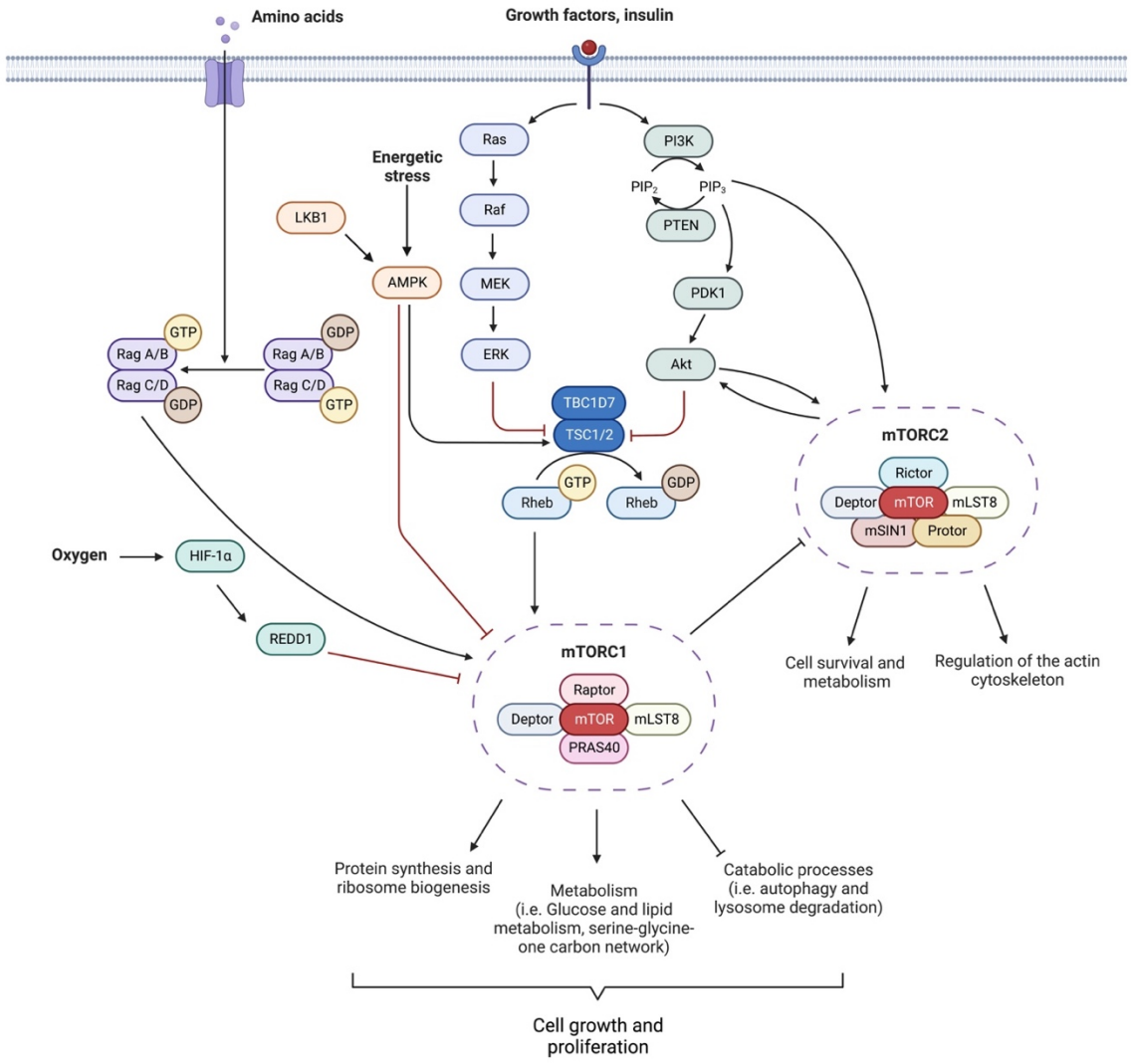


Fig 1.1 mTOR signaling pathway

The signaling pathways that regulate mTORC1 and mTORC2 are influenced by multiple inputs, including growth factors and hormones (e.g., insulin), amino acids, energetic status in the cell, and oxygen. These inputs are integrated into mTORC1 through complex mechanisms. When mTORC1 is active, it promotes cell growth and proliferation by stimulating anabolic processes such as protein, lipid, and nucleotide synthesis, as well as ribosome biogenesis, while inhibiting catabolic processes like autophagy and lysosome degradation (Saxton & Sabatini, 2017). Although the regulation of mTORC2 is not fully elucidated, there are clues pointing to interactions between mTORC1, mTORC2, and other signaling molecules (Saxton & Sabatini, 2017).

1.1. Upstream activators of mTOR signaling

Upstream activators of mTOR signaling, which are factors or pathways that directly or indirectly modulate mTOR activity, work together in a coordinated manner to regulate the activity of mTOR complexes (Fig 1.1). For example, insulin or insulin-like growth factor 1 (IGF-1) interact with their tyrosine kinase receptors (RTKs) on the plasma membrane, leading to activation of the phosphoinositide 3-kinase (PI3K)-phosphoinositide-dependent kinase 1 (PDK1)-AKT axis (Cohen et al., 1997; Hopkins et al., 2020; Ruderman et al., 1990). This signaling pathway is tightly regulated by the tumour suppressor protein phosphatase and tensin homolog (PTEN), which exerts negative control over the pathway (Neshat et al., 2001). The regulators that activate the mTORC1 pathway by way of the PI3K-PDK1-AKT axis achieve this by inhibiting the tuberous sclerosis complex (TSC), which is comprised of TSC1 and TSC2 (Inoki et al., 2002). This complex functions as a negative regulator of mTORC1 via GTPase-activating protein

(GAP) activity towards Ras homologue enriched in brain (RHEB) (Kwiatkowski & Manning, 2005). TBC1D7, a member of the TBC1 domain family, associates with the TSC1-TSC2 complex to modulate mTORC1 signaling. By interacting with TSC1-TSC2, TBC1D7 contributes to the regulation of mTORC1 signaling (Dibble et al., 2012). AKT activates mTORC1 by phosphorylating and inactivating the tumour suppressor TSC2, which bolsters the activity of RHEB (K. Inoki et al., 2003; Inoki et al., 2002).

At the same time, amino acid availability is a major regulator of mTORC1 (Takahara et al., 2020). When amino acids are introduced, the Rag GTPases undergo a change that causes them to enter their active nucleotide-bound state. Specifically, RagA or B becomes GTP-bound, while RagC or D becomes GDP-bound. In their active state, the Rag GTPases can bind Raptor, which enables them to attract mTORC1 to the lysosomal surface (Sancak et al., 2008). This is where RHEB is thought to be located, and since RHEB is a direct activator of mTORC1 (Saucedo et al., 2003), the association between mTORC1 and RHEB results in the activation of mTORC1 (Sancak et al., 2008). In addition, the Ras-Raf-MEK-ERK axis, activated by RTKs, regulates the activity of mTORC1 by phosphorylating TSC2, and leading to dissociation of the TSC1-TSC2 complex (Ma et al., 2005; Roux et al., 2004). Additionally, cellular energy status, as sensed by AMPK, can modulate mTOR signaling. When cellular energy is low, AMPK is activated, inhibiting mTORC1 both directly, through the phosphorylation of Raptor (Gwinn et al., 2008), as well as indirectly through the phosphorylation and activation of TSC2 (Ken Inoki et al., 2003). The tumour suppressor liver kinase B1 (LKB1) negatively regulates the activity of mTORC1 through activation of AMPK (Shaw et al., 2004; Stein et al., 2000; Woods et al., 2003). When DNA damage or hypoxia occurs, the protein called regulated in development and DNA damage response (REDD1) is induced. REDD1 then promotes the dissociation of inhibitory 14-3-3

proteins from TSC2, which ultimately results in the inhibition of mTORC1 (Dennis et al., 2014; DeYoung et al., 2008).

mTORC2 is primarily activated by insulin and other growth factors through PI3K. The latter is facilitated by the mSIN1 subunit which inhibits mTORC2 in the absence of insulin but activates it when insulin is present by binding to PIP3 (Dalle Pezze et al., 2012; P. Liu et al., 2015). AKT interacts with mTORC2. The phosphorylation of SIN1 at T86 by AKT results in an increase in mTORC2 kinase activity. This, in turn, leads to the phosphorylation of the hydrophobic motif of AKT (S473) by mTORC2, which ultimately triggers the full activation of AKT (G. Yang et al., 2015). mTORC2 promotes mTORC1 signaling through AKT-dependent phosphorylation of TSC2 and other effectors (Jacinto et al., 2006). However, mTORC2 is also regulated by mTORC1 through a negative feedback loop involving S6K-mediated degradation of IRS-1 and phosphorylation of GRB10 (Harrington et al., 2004; Hsu et al., 2011; Shah et al., 2004; Yu et al., 2011). The interconnectivity between mTORC1 and mTORC2 suggests cooperation between the two complexes in mediating metabolic processes, and potential drug targets in their feedback loops.

1.2. Functions of the mTOR signaling pathway

mTORC1 and mTORC2 are structurally and functionally divergent complexes (Loewith et al., 2002; Sarbassov et al., 2004). mTORC1 plays a pivotal role in regulating several cellular processes that influence cell growth and proliferation. These processes include protein synthesis, glucose and lipid metabolism, and nucleotide synthesis, as well as catabolic processes such as autophagy and lysosomal degradation (Saxton & Sabatini, 2017). mTORC2 is thought to regulate cytoskeleton organization, cell metabolism and survival via phosphorylating and

modulating activity of AGC protein kinases such as AKT and SGK1 (Saxton & Sabatini, 2017) (Fig 1.1).

1.2.1. Roles of mTORC1

1.2.1.1. Protein synthesis and ribosome biogenesis

mTORC1 plays a key role in regulating protein synthesis by controlling the activity of several downstream targets that are involved in the initiation, elongation, and termination phases of protein synthesis (Nandagopal & Roux, 2015). One of the key downstream targets of mTORC1 is the ribosomal protein S6K. When mTORC1 is activated, it phosphorylates S6K, which then phosphorylates several factors implicated in translation including ribosomal proteins like ribosomal protein S6 (rpS6) (Shima et al., 1998) and other factors such as eIF4B (Eukaryotic translation initiation factor 4B) (Holz et al., 2005). eIF4B is essential for recruiting ribosomes to the translation initiation complex and promoting the RNA helicase activity of eukaryotic translation initiation factor 4A (eIF4A), which unwinds mRNA for initiation of codon scanning (Ma & Blenis, 2009; Pelletier & Sonenberg, 2019). S6K1 not only phosphorylates PDCD4, an inhibitor of eIF4A helicase, but also facilitates its degradation, thus bolstering unwinding of secondary structures in 5'UTRs by eIF4A during ribosome scanning (Dorrello et al., 2006). It was also suggested that S6Ks may increase efficiency of translation of spliced mRNAs by interacting with an S6K1-specific interactor S6K1 Aly/REF-like substrate (SKAR), a component of exon-junction complexes (Ma et al., 2008). S6Ks also phosphorylate eEF2 kinase (eEF2K) at S366, causing a decrease in eEF2K activity towards eukaryotic translation elongation factor 2 (eEF2), which results in eEF2 dephosphorylation, and increase in its activity and elongation rates (Browne & Proud, 2004; Redpath et al., 1996; Wang et al., 2001).

Another important downstream target of mTOR is the 4E-BP family, which includes three members in mammals: 4E-BP1, 4E-BP2, and 4E-BP3. Of these, 4E-BP1 is the most extensively studied and best-characterized to date (Gingras, Raught, Gygi, et al., 2001; Heesom & Denton, 1999). 4E-BP1 can be phosphorylated at the NH2-terminal sites (Thr37/46 in human 4E-BP1) by mTOR which are required for subsequent modification of the sites adjacent to the eIF4E-binding site (Ser65/Thr70) (Gingras et al., 1999; Mothe-Satney et al., 2000). 4E-BPs bind to the eukaryotic initiation factor 4E (eIF4E), which binds 5' mRNA cap (Gingras, Raught, Gygi, et al., 2001). When 4E-BPs bind to eIF4E, they compete with eIF4G and prevent eIF4G from binding to eIF4E. This, in turn, inhibits the formation of the eIF4F complex, which plays a key role in cap-dependent translation (Gingras, Raught, & Sonenberg, 2001). Given this inhibitory role of 4E-BPs on mRNA translation, the inhibition of cell proliferation by 4E-BPs is achieved by specifically blocking the translation of mRNAs that encode proteins responsible for promoting proliferation and driving cell cycle progression, including cyclin D3 (Dowling et al., 2010). mTORC1 plays a crucial role in regulating ribosome biogenesis, which is the process of creating ribosomes, the cellular machinery responsible for protein synthesis. mTORC1 achieves this effect through phosphorylation and activation of downstream targets, such as S6K1 and transcription initiation factor 1A (TIF-1A) (Iadevaia et al., 2014). For example, S6K1 phosphorylates the carboxy-terminal activation domain of the nucleolar transcription factor UBF to regulate 45S ribosomal gene transcription (Hannan et al., 2003). In addition, the mTORC1/S6K1 axis plays a role in activating TIF-1A, an essential component of the RNA polymerase I (Pol I) complex responsible for driving rRNA transcription (Mayer et al., 2004). Overall, mTORC1 coordinates the different steps of protein synthesis and ribosome biogenesis

by regulating the activity of several downstream targets such S6Ks and 4E-BPs, leading to an increase in the rate of protein synthesis and cell growth.

1.2.1.2. Regulation of metabolism

The regulation of cellular metabolism is a pivotal function of mTORC1, as it helps to maintain a balance between anabolic and catabolic processes in response to varying levels of nutrients, energy, and oxygen availability (Saxton & Sabatini, 2017). One of the key functions of mTORC1 in metabolism is the regulation of glucose metabolism. mTORC1 can positively control the expression of transcription factors HIF1α and c-Myc (Dodd et al., 2015; K. Düvel et al., 2010; West et al., 1998). These transcription factors, in turn, can up-regulate the expression of several genes related to glucose uptake and glycolysis such as *GLUT1*, a glucose transporter, and phosphofructokinase (*PFK*), an enzyme involved in the glycolytic pathway (Buller et al., 2008; Minchenko et al., 2003). This leads to enhanced glucose uptake and glycolysis, thereby promoting increased flux through the glycolytic pathway, ultimately resulting in higher production of ATP, reducing equivalents, and building blocks for macromolecular synthesis. This aids in the integration of nutrients into new biomass by switching glucose metabolism from oxidative phosphorylation to glycolysis (Lunt & Vander Heiden, 2011).

mTORC1 also regulates lipid metabolism by promoting the synthesis of fatty acids and cholesterol, and by inhibiting lipolysis and fatty acid oxidation (Lamming & Sabatini, 2013). This is achieved through the activation of several downstream targets, such as sterol regulatory element binding proteins (SREBPs) (K. Düvel et al., 2010; Timothy R. Peterson et al., 2011), the peroxisome proliferator-activated receptor gamma (PPARγ) (Angela et al., 2016), and Lipin1 (T. R. Peterson et al., 2011). Although the activation of SREBPs is traditionally associated with low levels of sterols, recent studies have shown that mTORC1 signaling can also activate SREBPs

through two distinct mechanisms. Firstly, S6K1 is involved in activating SREBP (K. Düvel et al., 2010). Secondly, mTORC1 signaling can inhibit Lipin1 phosphorylation, which, in turn, suppresses SREBPs in the absence of mTORC1 signaling (Timothy R. Peterson et al., 2011). mTORC1-mediated activation of SREBPs facilitates increased flux via the oxidative pentose phosphate pathway (PPP), generating NADPH and other intermediary metabolites necessary for cell growth and proliferation (Katrin Düvel et al., 2010; Shimomura et al., 1998).

Furthermore, mTORC1 regulates serine biosynthesis and one-carbon pathway metabolism through transcriptional regulation, post-translational modifications, and SAMTOR-mediated sensing of SAM levels (Zeng et al., 2019). To this end, mTORC1 can activate transcription factors such as ATF4 to promote the expression of enzymes involved in serine biosynthesis and one-carbon metabolism (Adams, 2007; Ben-Sahra et al., 2016). Additionally, mTORC1 interacts with SAMTOR, which acts as a sensor for SAM levels. When SAM levels are high, SAMTOR dissociates from mTOR, allowing mTOR to become active and promote serine biosynthesis and one-carbon metabolism (Gu et al., 2017).

Recent research has demonstrated that mTORC1 is involved in promoting the synthesis of nucleotides necessary for DNA replication and ribosome biogenesis in rapidly growing and dividing cells (Saxton & Sabatini, 2017). To achieve this, mTORC1 enhances the expression of MTHFD2, a vital component of the mitochondrial tetrahydrofolate cycle, through ATF4-dependent pathways. MTHFD2 is responsible for supplying one-carbon units needed for purine synthesis (Ben-Sahra et al., 2016). Furthermore, S6K1 activates carbamoyl-phosphate synthetase (CAD), a critical enzyme involved in the de novo pyrimidine synthesis pathway, through phosphorylation (Ben-Sahra et al., 2013; Robitaille et al., 2013). Collectively, these mechanisms

highlight the role of mTORC1 in regulating these metabolic pathways, which play an indispensable role in cellular growth.

1.2.1.3. Repression of catabolic processes

mTORC1 has been traditionally associated with promoting anabolic processes such as protein synthesis, lipid synthesis, and nucleotide synthesis. However, recent studies have also shown that mTORC1 can regulate catabolic processes, including autophagy and lysosomal degradation. Autophagy is a process by which cells degrade and recycle damaged or unnecessary cellular components, such as misfolded proteins, damaged organelles, and intracellular pathogens (Mizushima & Komatsu, 2011). mTORC1 inhibits autophagy by phosphorylating and inhibiting Unc-51-like autophagy activating kinase 1 (ULK1) (Kim et al., 2011), which is required for the initiation of autophagy, and Atg13, which is a key regulator of autophagosome formation (Popelka & Klionsky, 2017). In addition, another proposed mechanism for mTORC1-mediated regulation of ULK1 involves the inhibition of ULK1 stability through phosphorylation of autophagy/beclin 1 regulator 1 (AMBRA1) by mTORC1 (Nazio et al., 2013).

mTORC1 also inhibits the activity of transcription factor EB (TFEB) that promotes the expression of genes involved in lysosomal biogenesis and function (Martina et al., 2012). On the other hand, mTORC1 promotes lysosomal degradation by regulating the activity of lysosomal enzymes and the trafficking of lysosomes to different subcellular compartments (Puertollano, 2014). mTORC1 regulates lysosomal enzymes through the activation of TFEB, which promotes the expression of genes involved in lysosomal function (Martina et al., 2012; Martina & Puertollano, 2013; Settembre et al., 2012). In addition, mTORC1 promotes the trafficking of lysosomes to the perinuclear region of the cell, where they can efficiently degrade cellular components (Hong et al., 2017). Overall, the function of mTORC1 in catabolism is to maintain a

balance between anabolic and catabolic processes in response to changes in nutrient availability and energy status. The role of mTORC1 in catabolism is complex and context-dependent, and its dysregulation has been implicated in several diseases, including neurodegenerative disorders, metabolic disorders, and cancer (Sabatini, 2017). Thus, understanding the regulation of mTORC1 in catabolism is important for the development of new therapeutic strategies to treat these diseases.

1.2.2. Roles of mTORC2

One of the key functions of mTORC2 is the regulation of cell survival and metabolism through the phosphorylation of several downstream targets, including AKT and serum- and glucocorticoid-regulated kinase 1 (SGK1) (García-Martínez & Alessi, 2008; Ikenoue et al., 2008; Sarbassov et al., 2005). AKT and SGK1 play critical roles in regulating cell survival, metabolism, and growth, by activating several downstream pathways, including the insulin signaling pathway and the regulation of ion transporters (Lang et al., 2006; Sugiyama et al., 2019). mTORC2 also plays a role in the regulation of the actin cytoskeleton, which is important for cell movement and shape change through the phosphorylation of AGC kinases including AKT, SGK1 and PKC (Jacinto et al., 2004; Senoo et al., 2019). Additionally, mTORC2 has been implicated in the regulation of glucose and lipid metabolism, although its precise role is still not fully understood. It has been suggested that mTORC2 may regulate glucose metabolism through the activation of AKT (Hagiwara et al., 2012; Yuan et al., 2012). In addition, it has been shown that mTORC2 primarily inhibits apoptosis through the AKT-mediated phosphorylation of Foxo1/3, Caspase 3, BCL2 associated agonist of cell death (BAD), and Bcl-2 interacting mediator of cell death (BIM) (Datta et al., 1997; Zhang et al., 2011; Zhou et al., 2000; Zhu et al., 2008). Overall, the task of mTORC2 is to regulate cell survival, metabolism, and cytoskeletal

organization through the phosphorylation of several downstream targets, mainly AKT and SGK1. Although its precise role in metabolism is still not fully understood, it is clear that mTORC2 plays a critical role in the regulation of cellular processes that are essential for normal cellular function and development.

1.3. mTOR inhibitors

1.3.1. Rapamycin and rapalogs

Rapamycin is a natural product that was first isolated from a soil sample collected on Easter Island in the 1970s (Sehgal et al., 1975). It is a macrolide compound that has immunosuppressive and antifungal properties (Abraham & Wiederrecht, 1996; Sehgal et al., 1975). It works by binding to FKBP12, which then associates with a specific domain on mTORC1 called the FKBP12-rapamycin binding domain (FRB). This binding event induces a conformational change in mTORC1 that inhibits its kinase activity (Abraham & Wiederrecht, 1996). Rapalogs are a class of drugs that are derived from the natural product rapamycin, and some examples of rapalogs include sirolimus, everolimus, and temsirolimus (Kuerec & Maier, 2023).

One of the limitations of rapamycin and rapalogs is that they are specific inhibitors of mTORC1 and do not inhibit mTORC2 activity (Feldman et al., 2009; Thoreen et al., 2009). In certain situations, this phenomenon can trigger a compensatory enhancement in mTORC2 signaling, which can paradoxically support cell survival and inhibit apoptosis (Tabernero et al., 2008). This could potentially elucidate why rapalogs primarily exhibit cytostatic effects on tumours, rather than cytotoxic effects (Rozengurt et al., 2014; Tabernero et al., 2008). Moreover, rapamycin does not fully inhibit all mTORC1 outputs, whereby it blocks the phosphorylation of S6Ks but not 4E-BPs (Choo et al., 2008) (Fig 1.2). Nonetheless, rapamycin and its rapalog derivatives remain an

important class of drugs in the treatment of cancer and other diseases, and ongoing research is focused on developing more effective mTOR inhibitors. For example, Everolimus, categorized as a rapalog, is employed in combination therapy for various chemoresistant cancers due to the frequent activation of the mTOR pathway in these cases (Forbes et al., 2011; Houghton, 2010). Several non-randomized studies conducted in HER2-positive trastuzumab-resistant metastatic breast cancer (mBC) have demonstrated the anti-tumor efficacy of including everolimus alongside standard chemotherapy and trastuzumab (Forbes et al., 2011; Jerusalem et al., 2011).

1.3.2. Active-site mTOR inhibitors, the second generation of mTOR inhibitors

Active-site mTOR inhibitors (asTORis) are drugs that directly inhibit the kinase activity of mTOR, by targeting its catalytic site (Liu et al., 2009; Yea & Fruman, 2013). These inhibitors bind to the ATP-binding pocket of mTOR, preventing the enzyme from phosphorylating downstream targets and reducing mTOR signaling. Unlike rapamycin, asTORi can inhibit all known outputs of both mTORC1 and mTORC2, leading to a more complete inhibition of mTOR signaling (Fig 1.2). Some examples of asTORi include Torin1, Ink128, AZD8055, and OSI-027 (Zheng & Jiang, 2015). To that effect, Ink128 has been used in various *in vivo* models including animal models of cancer, where it has demonstrated antitumour activity (Gökmen-Polar et al., 2012; Slotkin et al., 2015). Furthermore, Ink128 has been evaluated in clinical trials for the treatment of various types of malignancies, including solid tumours and lymphomas (Hsieh et al., 2012; Li et al., 2015; Pui et al., 2008). While Ink128 demonstrated cytotoxic effects (Hsieh et al., 2012), other reports have indicated its cytostatic effect (Janes et al., 2013), suggesting the potential for combining mTOR-targeted therapies with other anti-cancer agents. Furthermore, asTORis can also inflict off-target effects on other kinases and cellular processes, and their clinical development is still ongoing (Pallet & Legendre, 2013).

1.3.3. Third generation of mTOR inhibitors

The newer class of drugs, known as the third generation of mTOR inhibitors, is designed particularly to target cancer cells that have become resistant due to mutations in the FRB/kinase domain of the mTOR protein or compounds that selectively inhibit mTORC1 without affecting mTORC2 (Lee et al., 2021; Rodrik-Outmezguine et al., 2016). The first class includes RapaLink-1, a bivalent mTOR inhibitor that targets two drug-binding pockets: an allosteric site and a kinase site, in order to overcome resistance to existing first and second generation inhibitors (Rodrik-Outmezguine et al., 2016). The latter class includes a prototype inhibitor called Bis-35x, which belongs to a group known as 'bi-steric inhibitors." (Lee et al., 2021) It comprises a rapamycin-like central molecule connected chemically to an active site targeting inhibitor for the kinase. As a result, Bis-35x has demonstrated higher potency than other kinases in its capacity to block mTOR activity, while displaying selectivity towards mTORC1 (Lee et al., 2021) (Fig 1.2). In developing these inhibitors, overcoming resistance mechanisms that may emerge from acquired mutations within the cancer cells were foremost in mind, resulting in effective treatments (Rodrik-Outmezguine et al., 2016).

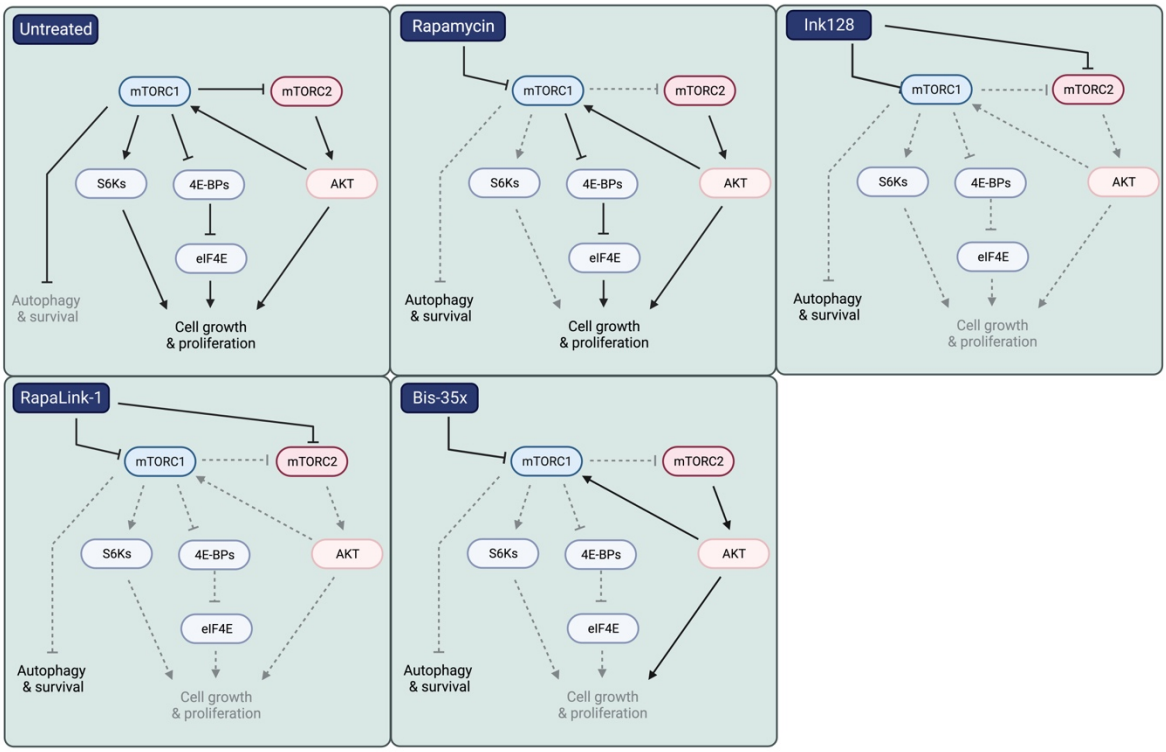


Fig 1.2. mTOR inhibitors

The figure is adapted from (Saxton & Sabatini, 2017). The use of first generation mTOR inhibitor rapamycin, second generation inhibitor Ink128, and third generation inhibitor RapaLink-1 and Bis-35x has diverse impacts on cancer cell growth and survival.

2. Epigenetics

Epigenetics refers to changes in gene expression or cellular phenotype that occur without altering the underlying DNA sequence (Bird, 2007). These changes can be inherited through cell division (Daxinger & Whitelaw, 2012; Morgan et al., 1999) and, in some cases, across generations, making epigenetic modifications an important mechanism for regulating gene expression and cellular functions (Berger et al., 2009). Epigenetic modifications can occur at various levels, such as DNA methylation, histone modifications, and via non-coding RNA molecules, and they can be influenced by a variety of factors, including environmental exposures, diet, and lifestyle (Alegría-Torres et al., 2011; Portela & Esteller, 2010). These modifications can lead to changes in gene expression and cellular behaviour that affect development, disease susceptibility, and aging (Zoghbi & Beaudet, 2016). Research in epigenetics has led to new insights into the mechanisms underlying various diseases, including cancer, cardiovascular disease, and neurodegenerative disorders (Cheng et al., 2019; Handy et al., 2011; Landgrave-Gómez et al., 2015). Furthermore, epigenetic modifications can potentially be targeted by drugs, rendering them a promising area of study for the development of new therapies (Miranda Furtado et al., 2019; Umehara, 2022).

2.1. Epigenetic modifications

2.1.1. DNA/RNA methylation

DNA methylation is an epigenetic modification that involves the addition of a methyl group to the 5-carbon position of cytosine residues in the DNA molecule (Robertson, 2005). DNA methyltransferases (DNMTs) are responsible for transferring methyl group from S-adenosylmethionine (SAM) to cytosine (Chiang et al., 1996). The most common form of DNA

methylation occurs at CpG dinucleotides, which are regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide (Bird, 1986). DNA methylation plays a crucial role in gene regulation (Illingworth & Bird, 2009; Weber et al., 2007). In general, DNA methylation is associated with gene repression, although the relationship between DNA methylation and gene expression can be complex and context-dependent (de Mendoza et al., 2022). In particular, DNA methylation at promoter regions of genes can block the binding of transcription factors and/or RNA polymerase, thereby preventing transcription. On the other hand, DNA methylation at gene bodies and enhancer regions can regulate gene expression by facilitating the binding of transcription factors and chromatin remodeling complexes (Jones, 2012; Weber et al., 2007). DNA methylation plays a major regulatory role in diverse cellular processes, such as embryonic development, transcriptional regulation, chromatin structure modulation, X chromosome inactivation, and chromosome stability maintenance (Brenet et al., 2011; Lee & Lee, 2012; Li et al., 2022; Lodde et al., 2009; Mohandas et al., 1981). Notably, DNA methylation has been implicated in a broad spectrum of diseases, including cancer and neurological disorders, underscoring its significance in maintaining normal cellular function. (Kaur et al., 2022; Robertson, 2005)

The intricate process of RNA methylation pertains to the post-transcriptional modification procedure whereby a methyl group (-CH3) is added to the nucleotide enveloping RNA molecules (Zhou et al., 2020). One of the most prevalent forms of RNA methylation is N6-methyladenosine (m6A), frequently observed in numerous types of messenger RNA and select non-coding RNA varieties within eukaryotic organisms (Desrosiers et al., 1974). Diversifying the RNA methylation landscape are a few additional variations, such as N1-methyladenosine (m1A), 5-methylcytosine (m5C), and ribose 2'-O-methylation (M. Zhang et al., 2021). The majority of

messenger RNA (mRNA) methylation, specifically the methylation of m6A, is carried out by the collaborative action of METTL3 and METTL14 within RNA methyltransferase complexes. METTL3 functions as the catalytic enzyme responsible for adding the methyl group, while METTL14 plays a role in facilitating the binding of the RNA substrate during the methylation process (Bokar et al., 1997; Śledź & Jinek, 2016; Wang et al., 2016). RNA methylation can be reversed by enzymes known as "erasers", including Fat Mass and Obesity-Associated Protein (FTO) and AlkB Homolog 5 (ALKBH5) (Gerken et al., 2007; Zheng et al., 2013). Extensive research has focused on elucidating the multifaceted role of RNA methylation in a diverse range of biological processes, spanning from immune response and cell differentiation to development, gene translation, and DNA damage response (Chen et al., 2019; Han et al., 2019; Li et al., 2017; Weng et al., 2018; Xiang et al., 2017; Xing et al., 2020). This has led to the exploration of RNA methylation as a potential therapeutic target for diseases including cancer and neurological disorders.

2.1.2. Histone modifications

Histone modifications are a series of chemical changes that occur on the histone proteins, which play a role in packaging and organizing DNA within the nucleus of a cell (Y. Zhang et al., 2021). These modifications, including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation, among others, can impact the structure and function of chromatin, which is the complex of DNA and histone proteins that make up chromosomes (Kouzarides, 2007). These modifications can dynamically influence gene expression by promoting or inhibiting the binding of transcription factors and other regulatory proteins to specific DNA regions (Dong & Weng, 2013). Moreover, it has come to light that histone modifications can influence RNA splicing (de Almeida et al., 2011) and various DNA processes, including repair (Stucki et al., 2005) and

replication (Unnikrishnan et al., 2010). Thus, histone modifications serve as a crucial mechanism of epigenetic regulation, influencing the manipulation and expression of DNA within cells.

2.1.2.1. Histone acetylation

Histone acetylation is a frequently observed histone modification, through the addition of an acetyl group to lysine residues on proteins that are associated with chromosomal packaging. The status of histone acetylation is regulated by two opposing groups of enzymes, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Berndsen & Denu, 2008; Haberland et al., 2009). The acetylation of histones leads to the relaxation of chromatin structure, which makes the DNA more accessible to regulatory proteins and transcription factors, resulting in increased gene expression (Brownell & Allis, 1996; Shahbazian & Grunstein, 2007). Moreover, the acetyl group on the histone creates a binding site for other proteins, such as bromodomain-containing proteins, which can recruit additional factors to the chromatin and further enhance transcription (Josling et al., 2012). However, histone acetylation is reversible and can be removed by the aforementioned HDACs, which remove the acetyl group from the lysine residue. This process results in a more condensed chromatin structure and decreased gene expression (Andrew J. Bannister & Tony Kouzarides, 2011).

2.1.2.2. Histone methylation

Histone methylation, a prominent post-translational modification, entails the addition of methyl groups primarily to lysine or arginine residues on histone proteins, and is recognized as a pivotal part of epigenetic reprogramming (Greer & Shi, 2012). This modification is carried out by enzymes called histone methyltransferases (HMTs), which transfer a methyl group from SAM to the lysine or arginine side chains of histone proteins. Histone methylation can be removed by enzymes called histone demethylases (HDMs), which remove the methyl group from the lysine

or arginine residues (Kouzarides, 2007). Lysines can be subject to mono-, di-, and trimethylation, and the specific site of lysine and the degree of methylation each dictate distinct functions (A. J. Bannister & T. Kouzarides, 2011). Certain histone methylation marks, such as histone H3 lysine 4 (H3K4), histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) methylation, have been extensively studied and are known to be associated with specific transcriptional states, such as active, repressed, or poised (Black et al., 2012) (Fig 1.3). H3K4 methylation is widely regarded as a mark of transcriptional activation. While H3K4me1 is highly enriched at enhancers, H3K4me3 is commonly found at active promoters near transcription start sites (TSSs) (Liang et al., 2004; Sims et al., 2003). On the other hand, gene repression is commonly linked to the methylation of H3K9 and H3K27 (Barski et al., 2007). Several studies have reported the association between H3K9 methylation and gene silencing, including the inactive X chromosomes in both female mice and humans, as well as developmentally regulated genes (Heard et al., 2001; Litt et al., 2001; Nakayama et al., 2001; Peters et al., 2002). H3K9me2/3 is typically regarded as a marker of heterochromatin, influenced by the lysine HMT SUV39H1/2 and recognized by the chromodomain of heterochromatin protein-1 (HP1), thereby influencing the condensation of heterochromatic regions (Bannister et al., 2001; Rea et al., 2000). On the other hand, H3K9me1, primarily concentrated in the 5' UTR (Barski et al., 2007), is suggested to serve as a mediator between gene activation and repression by undergoing rapid methylation or demethylation (Black & Whetstine, 2011). Additionally, it has been observed that H3K9me3 is associated with the highly condensed centromeric (heterochromatic) regions of chromosomes (Peters et al., 2003). Mouse models with ablated modifiers for H3K4, H3K9, and H3K27 methylation have revealed the indispensable roles of histone methyltransferases in the regulation of transcription, maintenance of genome integrity, and embryonic development (Dodge et al.,

2004; O'Carroll et al., 2001; Peters et al., 2001; Rayasam et al., 2003). Therefore, understanding the regulation and function of histone methylation is essential for gaining insights into the mechanisms of gene expression and how they are perturbed in various diseases, including cancer, intellectual disability, and aging (Greer & Shi, 2012; Iwase & Shi, 2011; Peters et al., 2001; Pollina & Brunet, 2011). Furthermore, histone methylation is reversible and can be modified by various enzymes, rendering it a potential target for therapeutic interventions (Basavarajappa & Subbanna, 2021; Chen et al., 2020; Yang et al., 2021).

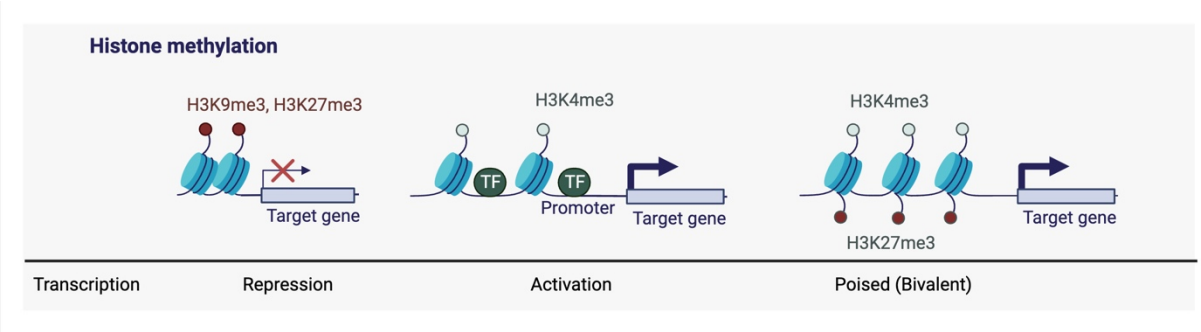


Fig 1.3. Histone methylation

The addition of methyl groups to histones can result in the formation of different histone methylation marks, such as H3K9me3 and H3K27me3, which are generally associated with gene repression. On the other hand, H3K4me3 is known to be involved in gene expression by facilitating the recruitment of transcription factors to promoter regions. Furthermore, a bivalent mark exists, merging the H3K27me3 modification associated with silencing and the H3K4me3 signal linked to activation, ultimately resulting in poised transcription.

2.1.2.3. Other histone post-translational modifications

Post-translational modifications, such as phosphorylation, sumoylation, ubiquitination and ADP-ribosylation, can also take place on histone proteins (Andrew J. Bannister & Tony Kouzarides, 2011). Histone phosphorylation involves the addition of a phosphate group to serine, threonine, or tyrosine residues on the histone protein (North et al., 2014). The latter is a dynamic process, with a well-established function observed during the cellular response to DNA damage. The addition of phosphate molecules onto histone H2A(X) causes vast areas within the chromatin to envelop the area where DNA damage has occurred. Such domains constitute a unique symbol for handling DNA damage response (van Attikum & Gasser, 2005). Histone sumoylation involves the addition of a small protein called small ubiquitin-like modifier (SUMO) to lysine residues on the histone protein (Nathan et al., 2006). This alteration impacts both the configuration of chromatin and the expression of genetic material. Additionally, it plays a prominent role in the management and maintenance of DNA repairing and replication processes (Ryu & Hochstrasser, 2021). Histone ubiquitination refers to the addition of ubiquitin to specific lysine residues on histone proteins. Notably, this modification has been identified on H2A (K119) and H2B (K20 in human and K123 in yeast) (Goldknopf et al., 1975; West & Bonner, 1980). In the human polycomb complex, the BMI/RING1A protein is responsible for mediating the ubiquitylation of H2AK119, which is associated with transcriptional repression (Wang et al., 2004), whereas H2BK123ub1 mediated by RAD6 plays an important role in transcriptional initiation and elongation (J. Kim et al., 2009; J. S. Lee et al., 2007). Histones can be subjected to mono- and poly-ADP ribosylation at specific glutamate and arginine residues, a process mediated by enzymes called Poly (ADP-ribose) polymerases (PARPs) (Hassa et al., 2006). Though the precise functional consequences of this modification are not yet fully understood and necessitate

further research, it has been suggested that poly-ADP ribosylated histones are associated with a more relaxed chromatin state, and the interplay between PARPs and histone ADP ribosylation is thought to play a role in regulating chromatin structure (Krishnakumar & Kraus, 2010). Overall, these post-translational modifications add a layer of complexity to the regulation of gene expression and chromatin structure.

3. Epigenetic modifications and related factors that are the subject of this thesis

3.1. H3K27 methylation

H3K27 methylation refers to the histone modification in which methyl groups are added to the lysine 27 residue of the histone H3 protein, resulting in H3K27me1, H3K27me2, and H3K27me3. This modification is associated with gene repression and plays an important role in development, differentiation, and cellular identity (Bernstein et al., 2006; Margueron et al., 2005; Pan et al., 2007; Simon & Kingston, 2009). H3K27 methylation is catalyzed by the Polycomb Repressive Complex 2 (PRC2), which includes the enzymatic subunit EZH2 (Cao et al., 2002). Histone methylation marks, such as H3K27me3, can be reversed by demethylases. To that end, two enzymes containing Jumonji domains, which possess demethylase activity specific for H3K27, have been identified. These enzymes are commonly referred to as ubiquitously transcribed tetratricopeptide repeat X (UTX, also known as KDM6A) and Jumonji domain-containing protein 3 (JMJD3, also known as KDM6B) (Klose & Zhang, 2007; Y. Xiang et al., 2007) (Fig 1.4). Recent research has suggested that the mTOR signaling pathway may regulate H3K27me3 levels, potentially through interactions with the PRC2 complex or other histone-modifying enzymes by regulating SAM or α-KG levels (Harachi et al., 2020; Morita et al., 2013;

Smith et al., 2019). This implies that H3K27me3 may play a role in linking cellular nutrient and energy sensing pathways to epigenetic regulation of gene expression.

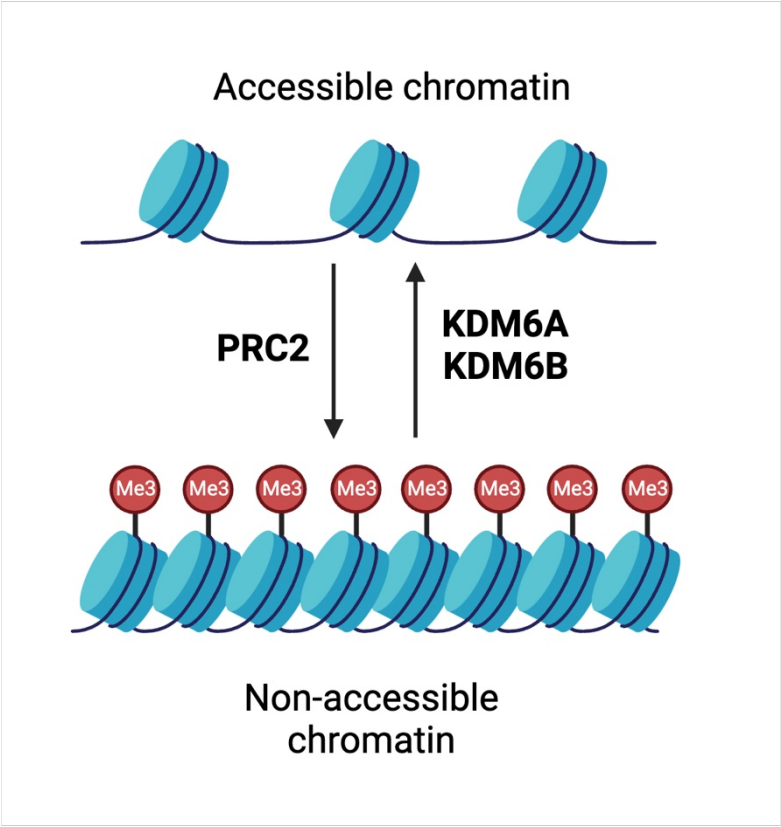


Fig 1.4. Epigenetic modifiers of H3K27me3

The addition of trimethylation to H3K27 is mediated by PRC2, while the removal of this methylation mark is catalyzed by KDM6A and KDM6B.

3.2. H3K27 methyltransferases – PRC2

The term "Polycomb" originated from a drosophila mutant that exhibited abnormal body segmentation, indicating that Polycomb likely functions as a negative regulator of homeotic genes involved in segmentation (Lewis, 1978). Currently, the Polycomb group (PcG) refers to a group of genes that share similar phenotypic effects with the original Polycomb mutant when mutated or disrupted (Blackledge et al., 2015; Kassis et al., 2017). The Polycomb system is composed of two multi-protein complexes, PRC1 and PRC2. PRC1 is an E3 ubiquitin ligase that mono-ubiquitylates histone H2A at lysine 119 (H2AK119ub1), and PRC2 is a histone methyltransferase that can monomethylate, dimethylate or trimethylate histone H3 at lysine 27 (H3K27me1, H3K27me2 or H3K27me3) (Piunti & Shilatifard, 2021). Mammalian PRC2 contains four core subunits: Enhancer of zeste homolog 1/2 (EZH1/2), Suppressor of zeste 12 homolog (SUZ12), Embryonic ectoderm development (EED) and Retinoblastoma-binding protein 4 or 7 (RBBP4/7) (Cao et al., 2002; Chen et al., 2018; Czermin et al., 2002; Pasini et al., 2004; Piunti & Shilatifard, 2021). EZH1, a close homolog of EZH2, is a component of a noncanonical PRC2 complex (Shen et al., 2008). It is reported that the catalytic activity of PRC2 is dependent on the SET domain of EZH1/2 (R. Margueron et al., 2008). However, EZH1/2 alone does not exhibit detectable HMT activity, and the presence of both EED and SUZ12 is essential for the HMT activity of PRC2 (Cao & Zhang, 2004; R. Margueron et al., 2009; Pasini et al., 2004). In addition, RBBP4 is important in bridging PRC2 and chromatin together by directly binding to the nucleosome (Cao & Zhang, 2004; Nekrasov et al., 2005). The activity of PRC2 is regulated through multiple mechanisms, which include accessory components within PRC2, post-translational modifications, substrate availability, and histone modifications (Fig 1.5).

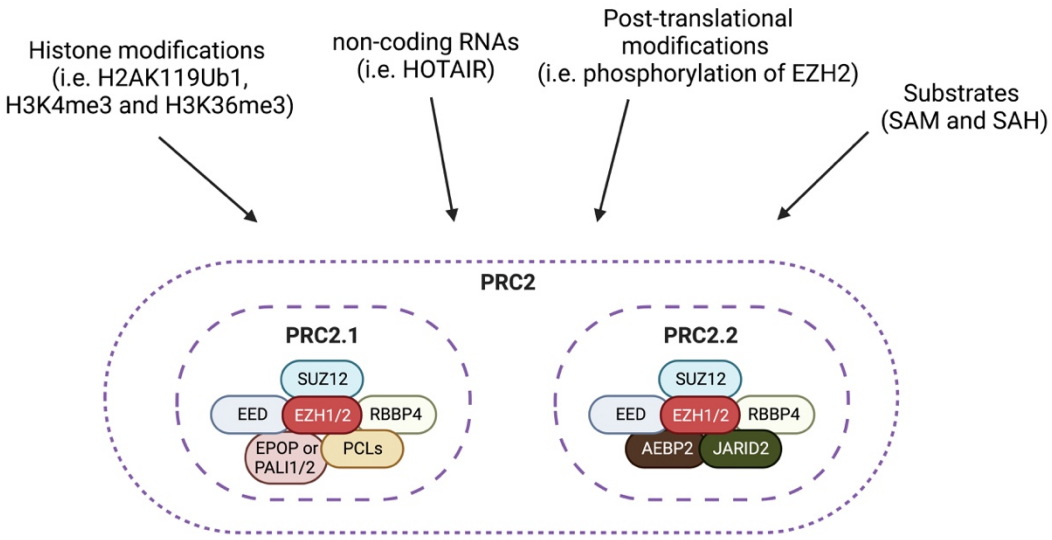


Fig 1.5. The components of PRC2.1 and PRC2.2 and the regulators of these complexes

PRC2 is composed of core members (EZH2, EED, SUZ12, and RBBP4) and facultative subunits, which form two distinct types of complexes known as PRC2.1 and PRC2.2. PRC2.1 contains PCLs (PHF1, MTF2, or PHF19) and either EPOP or PALI1/2, whereas PRC2.2 includes AEBP2 and JARID2 in association with the core subunits. The enzymatic activity of the core complex is stimulated by high-affinity binding of EED to the H3K27me3 mark, which may also occur in PRC2.1 and PRC2.2, although this has not been confirmed. The activity of these complexes can be regulated by various factors, including other histone modifications, non-coding RNAs, post-translational modifications, and substrate levels.

3.2.1. The regulation of PRC2 activity via its accessory components

Proteomic analyses have uncovered the existence of two distinct subtypes of PRC2, referred to as PRC2.1 and PRC2.2. PRC2.1 is composed of one of the Polycomb-like proteins (PCLs), such as PHD finger protein 1 (PHF1), Metal response element binding transcription factor 2 (MTF2), or PHD finger protein 19 (PHF19), along with either PALI1/2 or Elongin BC and polycomb repressive complex 2-associated protein (EPOP). On the other hand, PRC2.2 includes Adipocyte enhancer-binding protein (AEBP2) and Jumonji- and AT-rich interaction domain containing 2 (JARID2) (Yang & Li, 2020) (Fig 1.5). Although these additional subunits are not strictly essential for core PRC2 formation, their presence can impact PRC2 recruitment and catalytic activity (Højfeldt et al., 2019). For instance, deletion of individual PCLs had a mild effect on PRC2 recruitment, whereas triple knockout of PCL paralogues resulted in a significant reduction in PRC2 occupancy and H3K27me3 deposition (Healy et al., 2019).

Studies have revealed that JARID2 and AEBP2, when interacting with the core subunits of PRC2, exhibit functional similarities to the histone tail of H3 (Kasinath et al., 2018). The methylation of JARID2 at Lys116 by PRC2 has been shown to allosterically stimulate the activity of PRC2, mimicking a similar allosteric activation that is promoted by the interaction between H3K27me3 and EED (Kasinath et al., 2018; Sanulli et al., 2015; Son et al., 2013). AEBP2 binds to the β-sheet-rich domain of SUZ12 and interacts with the region of RBBP4 that interacts with unmodified H3K4, potentially bypassing the inhibitory effect of H3K4me3 on PRC2 catalytic activity (Kasinath et al., 2018). AEBP2 also promotes PRC2 binding to nucleosomes *in vitro*, potentially enhancing the catalytic activity of the complex (H. Kim et al., 2009).

EZHIP, also known as catalytic antagonist of Polycomb (CATACOM), is a tissue-specific facultative subunit that is found in both the PRC2.1 and PRC2.2 protein complexes. EZHIP has been found in fusion proteins with MBTD1 and functions as a catalytic antagonist of Polycomb (Jain et al., 2019; Ragazzini et al., 2019). While EZHIP does not affect the recruitment of PRC2 to chromatin (Ragazzini et al., 2019), it inhibits PRC2 activity, albeit via a mechanism that has not been fully investigated (Hübner et al., 2019; Piunti et al., 2019). It has been proposed that the C-terminal region of EZHIP harbours a conserved sequence similar to the substitution of methionine for lysine at position 27 (K27M), thereby obstructing the catalytic function of PRC2 (Hübner et al., 2019; Jain et al., 2019). However, alternative mechanism has also been proposed whereby EZHIP may reduce the interaction between the core subunit and facultative subunits (such as AEBP2 and JARID2), thus limiting their ability to stimulate PRC2 enzymatic activity (Ragazzini et al., 2019). Overexpression of EZHIP transgenes results in a genome-wide reduction in H3K27me3 (Jain et al., 2019), whereas removal of EZHIP leads to ectopic enrichment of H3K27me3 in chromatin, with minimal impact on SUZ12 deposition (Ragazzini et al., 2019). These studies portray an intricate interplay among the various components of PRC2, and that any changes or alterations in these components can impact the activity and function of PRC2.

3.2.2. The regulation of PRC2 activity via post-translational modifications (PTMs)

The regulation of PRC2 components involves several PTMs such as methylation, phosphorylation, acetylation, ubiquitination, sumoylation and O-GlcNAcylation (Yang & Li, 2020). Recent advancements in research have unveiled that PRC2, in addition to its well-known role in catalyzing the methylation of H3K27, can also methylate a wide array of non-histone proteins, including its own subunits (Ardehali et al., 2017; He et al., 2012). EZH2 can be

automethylated and this automatic methylation occurs before the allosteric activation of PRC2, but is dispensable for the recruitment of PRC2 to chromatin (Lee et al., 2019; Wang et al., 2019). Phosphorylation is a prevalent PTM that regulates the catalytic activity and chromatin targeting of PRC2 subunits. Notably, phosphorylation of distinct sites on PRC2 subunits by various protein kinases can have different effects on PRC2 function (Li et al., 2020; Yang & Li, 2020). Firstly, EZH2 phosphorylation regulates PRC2 HMT activity. For example, AKT can phosphorylate EZH2 at the S21 site. When EZH2 S21 is phosphorylated by AKT, it displays lower catalytic activity, resulting in reduced global H3K27me3 levels (Cha et al., 2005). Additionally, phosphorylation at specific sites, such as T372 by P38α and T345/T416 by cyclin-dependent kinase 1/2 (CDK1/2), is imperative for the targeting of PRC2 to specific genomic loci (Kaneko et al., 2010; Palacios et al., 2010; C. C. Yang et al., 2015). Next, the binding of EZH2 to other components of PRC2 can be affected by its phosphorylation. For example, when EZH2 is phosphorylated at specific sites such as T311 by AMPK, Y244 by JAK3, or T487 by CDK1, it can disrupt its association with EED or SUZ12. As a result, the methyltransferase activity of PRC2 may decrease (Wan et al., 2018; Wei et al., 2011; Yan et al., 2016). Additionally, phosphorylation of EZH2 at various sites, such as T345 and T487 by CDK1, Y641 by JAK3, and T261 by CDK5, has been demonstrated to trigger subsequent ubiquitination and degradation of EZH2, revealing a regulatory mechanism for controlling EZH2 levels through phosphorylation-mediated proteolysis (Sahasrabuddhe et al., 2015; Wu & Zhang, 2011). Fig 1.6 provides additional information on PTMs of EZH2.

In addition to EZH2, other components of PRC2 can also undergo phosphorylation, which can impact PRC2 activity. For instance, SUZ12 can be phosphorylated by mitotic polo-like-kinase 1 (PLK1) at specific sites such as S539, S541, and S546, leading to regulation of its binding to

EZH2. Furthermore, phosphorylation at these sites can also promote ubiquitin-mediated degradation of SUZ12, indicating that SUZ12 phosphorylation plays a role in the assembly of PRC2 (Zhang et al., 2015). Similarly, in Drosophila, phosphorylation of the N-terminus of ESC (a mammalian EED homologue) by casein kinases 1/2 (CK1/2) results in homodimerization. This phosphorylation event is crucial for the formation and stability of a larger PRC2 complex that includes PCLs and histone deacetylase RPD3 (Tie et al., 2005). Overall, different PTMs may have distinct effects on PRC2, with methylation likely regulating catalytic activity, ubiquitination potentially influencing stability and assembly, and phosphorylation bearing complex and context-dependent effects. As described, a single PTM such as phosphorylation can yield distinct consequences when present on various PRC2 subunits or even on different sites of the same subunit.

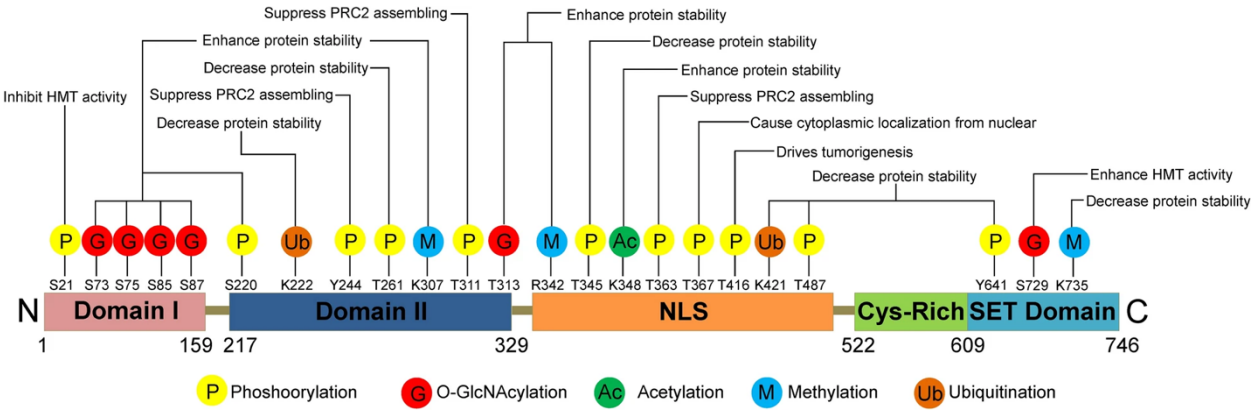


Fig 1.6. EZH2 PTMs

Figure from (Li et al., 2020). EZH2, a component of PRC2, undergoes different types of modifications (phosphorylation, O-GlcNAcylation, acetylation, methylation, ubiquitination),

shown in a plot with different colors. These modifications have various functions, indicating the diverse regulatory roles of EZH2 in gene expression and epigenetic regulation.

3.2.3. The regulation of PRC2 activity via histone modifications and non-coding RNAs

Histone modifications within the chromatin region play a major role in modulating the activity and binding of PRC2. For example, H3K27me3, a product of PRC2's own catalytic activity, has been observed to interact with the aromatic cage of EED. This interaction leads to the allosteric activation of PRC2, which in turn facilitates further deposition of H3K27me3 (Hansen et al., 2008; Raphael Margueron et al., 2009). Another example is the catalytic product of PRC1, H2AK119ub1, which can interact with JARID2 to facilitate PRC2 recruitment and H3K27me3 deposition (Cooper et al., 2014; Cooper et al., 2016). Other histone modifications, including H3K4me3 mediated by the MLL/COMPASS family proteins (Shilatifard, 2012) and H3K36me3 regulated by methyltransferase HYPB/Setd2 (Edmunds et al., 2008), can also impact the activity of PRC2 (Yang & Li, 2020). Specifically, H3K4me3 and H3K36me3 can inhibit the enzymatic activity of PRC2 *in vitro* (Schmitges et al., 2011). PRC2 facultative subunits PLCs can selectively bind to H3K36me3, thereby inhibiting PRC2 activity without affecting chromatin binding (Yuan et al., 2011). Moreover, PHF19, a component of PRC2.1, can bind to H3K36me3 and interact with H3K36me3 demethylase NO66 (Brien et al., 2012) and KDM2B (Ballaré et al., 2012), while PRC2 interacts with H3K4me3 demethylase RBP2 (JARID1A/KDM5A), facilitating the removal of H3K36me3 and H3K4me3, and promoting the deposition of H3K27me3 (Pasini et al., 2008). Overall, histone modifications have a significant impact on controlling the activity and binding affinity of PRC2 and are involved in the conversion of genes from an actively transcribing state to a state of polycomb-mediated repression.

3.2.4. The regulation of PRC2 activity via substrates

SAM is widely recognized as the universal methyl donor for methyltransferases, which transfer its methyl group to produce S-adenosylhomocysteine (SAH) and a methylated substrate (Finkelstein, 1990). This methylation process creates a connection between the cellular metabolism that regulates SAM and SAH levels, which may influence the epigenetic status of cells through product inhibition of methyltransferases (Serefidou et al., 2019) (Fig 1.7). SAM and SAH levels are instrumental in regulating PRC2 activity (Fioravanti et al., 2018). As a result, several inhibitors of EZH2, a key component of PRC2, have been developed by targeting SAM or SAH (Duan et al., 2020). For example, the first EZH2 inhibitor, 3-deazaneplanocin A (DZNep), acts as an inhibitor of SAH hydrolase, indirectly inhibiting EZH2 by increasing SAH levels, which in turn represses SAM-dependent histone methyltransferase activity (Miranda et al., 2009). Additionally, several potent and highly selective inhibitors of EZH2 that compete with SAM have been developed, such as GSK126 (GSK2816126) (Michael T. McCabe et al., 2012). GSK126 can effectively inhibit both wild-type and Y641 mutant EZH2 with similar potency and exhibits high selectivity compared to EZH1 (150-fold increased potency) or other methyltransferases (>1000-fold selective for EZH2) (Michael T. McCabe et al., 2012). UNC1999 represents the pioneering orally bioavailable inhibitor, demonstrating significant in vitro effectiveness against both wild-type and mutant forms of EZH2, along with EZH1 (Xu et al., 2015).

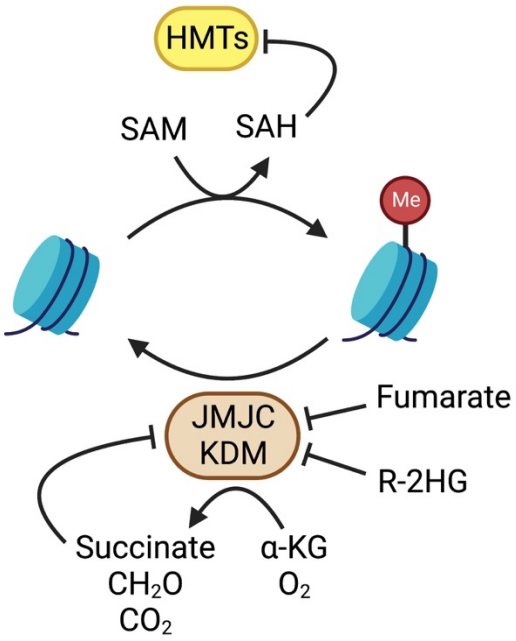


Fig. 1.7. The effect of metabolites in histone methylation and demethylation

Figure is adapted from (van der Knaap & Verrijzer, 2016). Histones are methylated by histone methyltransferases (HMTs), which require SAM as a substrate. The Jumonji C (JmjC) domain-containing histone lysine demethylases (KDMs) use α-KG as a cofactor. Fumarate, succinate, and R-2HG act as competitive inhibitors.

3.3. H3K27M mutation

The H3K27M mutation, which is a genetic alteration that results in the substitution of methionine for lysine at position 27 of histone 3, has been associated with poor prognosis in mainly pediatric and adult cases of diffuse intrinsic pontine glioma (DIPG) (El-Hashash, 2021). This mutation most commonly occurs in the H3.1 (HIST1H3B/C) or H3.3 (H3F3A) histone gene in these tumours (Castel et al., 2015; Chan et al., 2013; Meyronet et al., 2017; Wu et al., 2012).

The H3K27M mutation disrupts the normal function of PRC2 through an as-yet-unidentified molecular mechanism (Harutyunyan et al., 2020; Ashot S. Harutyunyan et al., 2019). *In vitro* studies have demonstrated that H3K27M strongly impacts the enzymatic activity of EZH2. This effect may be due to the high affinity binding of the enzyme to nucleosomes containing H3K27M, resulting in sequestration and inactivation of the PRC2 complex (Justin et al., 2016). This interference not only prevents the addition of trimethylation marks on mutant histones, but it also inhibits the "spreading" of H3K27me3 to wild-type histones across the genome. As a result, there is a global loss of H3K27me3 marks, accompanied by an increase in H3K27 acetylation (H3K27ac), leading to aberrant gene activation (Harutyunyan et al., 2020; Ashot S. Harutyunyan et al., 2019).

3.4. Functional roles of PRC2

The malfunctioning of PRC2 is a common characteristic of various human conditions, such as developmental disorders and cancer (Liu & Liu, 2022). PRC2 plays a crucial role in the regulation of gene expression associated with pluripotency and differentiation (Margueron & Reinberg, 2011). During the process of lineage commitment, cells need to turn off genes that are not necessary for their particular cell type, and the PRC2 complex participates in this by inhibiting the expression of these genes (Boyer et al., 2006; Chamberlain et al., 2008; O'Carroll et al., 2001; Riising et al., 2014). Studies on mouse embryonic stem cells have shown that the removal of SUZ12, JARID2 or PCL2 impairs the ability of PRC2 to silence the pluripotency factors like NANOG and OCT4, indicating that PRC2 is necessary for proper repression of these factors during differentiation (Li et al., 2010; Pasini et al., 2007; Walker et al., 2011).

Dysregulation of PRC2 activity is associated with various diseases, including cancer (Celik et al., 2018; Kleer et al., 2003; Varambally et al., 2002; Y. Wu et al., 2018). EZH2 demonstrates elevated expression and/or amplification of its locus in various cancer types, which correlates with an unfavorable prognosis (Barsotti et al., 2015; Shen et al., 2013; Varambally et al., 2002). Notably, EZH2 inactivation through loss-of-function mutation or PTM is also observed in myeloid malignancies and acute myeloid leukemia (Ito et al., 2018; Nakamura et al., 2014), Depending on the EZH2 status, EZH2 can have both anti-tumorigenic and pro-tumorigenic effects. PRC2-mediated H3K27me3 can also silence genes that are important for cell cycle regulation (Jacobs et al., 1999), DNA repair (Campbell et al., 2013), and apoptosis (Tan et al., 2007), allowing cancer cells to proliferate uncontrollably and resist apoptosis. On the other hand, the loss of EZH2 results in a reprogramming of BCAA metabolism, promoting leukemic transformation (Gu et al., 2019). The H3K27M mutation, considered a molecular driver of diffuse intrinsic pontine glioma (DIPG) (Moch et al., 2016), plays an essential role in early development, cell proliferation, and neoplastic transformation, indicating the critical involvement of H3K27me3 in regulating these cellular functions (Ashot S. Harutyunyan et al., 2019; Kfoury-Beaumont et al., 2022).

Therefore, targeting H3K27me3 represents a promising therapeutic approach for cancer treatment, particularly for cancers that are driven by aberrant gene silencing (Duan et al., 2020; Wei Qi et al., 2017). However, clinical trials will be needed to determine the safety and efficacy of these therapies in humans, and to identify the most effective combinations of treatments for specific types of cancer (Huang et al., 2018). For instance, the FDA granted approval for the EZH2 inhibitor tazemetostat (Tazverik; Epizyme) designed for the treatment of follicular lymphoma (FL) and epithelioid sarcoma (ES) (Mullard, 2020). This drug has demonstrated its

efficacy in the treatment of advanced solid tumors and lymphomas that carry EZH2-activating mutations, all while maintaining acceptable tolerability (Italiano et al., 2018; Ribrag et al., 2018). Currently, clinical trials are also underway to investigate other EZH2 inhibitors, such as CPI-1205, PF-06821497, and Valemetostat Tosylate (M.-L. Eich et al., 2020; Mullard, 2020). In some cases, targeting H3K27me3 alone may not be sufficient to effectively treat neoplasia (M. L. Eich et al., 2020). Nevertheless, combining H3K27me3-targeted therapies with other treatments, such as chemotherapy or immunotherapy, may enhance their effectiveness (Duan et al., 2020). For example, a recent study showed that combining tazemetostat,with the immunotherapy drug pembrolizumab was effective in treating patients with advanced solid tumours (Shin et al., 2022).

3.5. H3K27 demethylases – KDM6A/B

H3K27 demethylation is catalyzed by two enzymes, KDM6A and KDM6B (Klose & Zhang, 2007; Y. Xiang et al., 2007). KDM6A/B is a member of the JmjC domain-containing histone demethylase family (Hong et al., 2007) and specifically removes the H3K27me3 catalyzed by PRC2, resulting in the increase of gene expression by removing the repressive H3K27me3 mark from target genes (Agger et al., 2007; De Santa et al., 2007; M. G. Lee et al., 2007). During development, H3K27 demethylases help guide the differentiation of stem cells into different cell types by activating lineage-specific genes (Swigut & Wysocka, 2007). For instance, ectopic expression of JMJD3 results in a significant reduction of H3K27me3 levels and leads to the displacement of polycomb proteins *in vivo* and UTX directly binds to the HOXB1 locus and is necessary for its activation, which is consistent with the marked decrease in H3K27me3 levels associated with HOX genes during the process of differentiation (Agger et al., 2007).

KDM6A and KDM6B play complex roles in cancer initiation and progression, and their functions vary depending on the specific subtype of cancer and the context in which they are expressed. KDM6A is often mutated and inactivated in multiple cancer types such as multiple myeloma, non-small cell lung cancer and colorectal cancer (Ezponda et al., 2017; Q. Wu et al., 2018; Zha et al., 2016). In breast cancer, however, KDM6A has been shown to have both low and high expression associated with poor prognosis (Taube et al., 2017; Xie et al., 2017; Yu et al., 2019). KDM6B was also shown to mediate carcinogenic and anti-cancer signaling pathways in a context-dependent manner. It can act as a tumour suppressor in some cancers by downregulating different transcriptional programs such as NEFM (Yang et al., 2019) and p15INK4B (R. Tokunaga et al., 2016), while it can act as an oncogene in other cancers by promoting proliferation (McLaughlin-Drubin et al., 2013; Ohguchi et al., 2017), survival (Zhang et al., 2016), migration, stem cell behavior, epithelial-to-mesenchymal transition (EMT) (Tang et al., 2016; Wang et al., 2021), drug resistance (D'Oto et al., 2021; Wang et al., 2018), and tumour microenvironment (Nagarsheth et al., 2016; Park et al., 2016).

Given the foregoing, and the fact that overexpression of KDM6 demethylases is observed in various cancers (Yang Xiang et al., 2007; Xie et al., 2017), targeting KDM6A and KDM6B with enzymatic inhibitors constitutes a potentially attractive therapeutic strategy (Morozov et al., 2017; Zhang et al., 2020). Since KDM6A/B requires Fe (II) as a cofactor and α-ketoglutarate (α-KG) as a co-substrate for catalyzing reactions, most inhibitors of KDM6 demethylases work by binding competitively with α-KG at the active site of the JmjC domain and chelating the Fe (II) residue (Tricarico et al., 2020) (Fig 1.7). Notably, in a specific subset of human cancers such as paragangliomas (PGLs) and renal cell carcinomas, loss-of-function mutations in succinate dehydrogenase (SDH) and fumarate hydratase (FH) are reported (Burnichon et al., 2010; Castro-

Vega et al., 2014; Moch et al., 2016). These mutations result in the accumulation of fumarate and succinate, which share structural similarity with α-KG and subsequently inhibit various α-KG-dependent dioxygenases, including KDM6A and KDM6B (Cervera et al., 2009; Smith et al., 2007; Xiao et al., 2012).

4. Rationale

Although the role of mTOR in various biological processes – such as protein synthesis, metabolic regulation, and autophagy – has been widely investigated, its impact on epigenetic reprogramming remains largely unknown. Histone methylation, unlike other epigenetic modifications such as DNA methylation or histone acetylation, is a complex and dynamic process that occurs at multiple sites on the histone tail and bears diverse effects on gene expression. Studying histone methylation can provide valuable insights into the impact of epigenetic regulation on cellular processes and may offer new therapeutic approaches for diseases like cancer, where histone methylation dysregulation is common.

Recent studies reported that mTOR may govern histone methylation through its regulation of epigenetic modifiers such as EZH2, SUZ12 and G9a (Arjamand Mushtaq et al., 2023; Smith et al., 2019). For instance, c-Src oncogene has been shown to alleviate energy stress, thereby allowing for sustained activation of mTORC1, which in turn increases the translation of mRNAs that encode the subunits of the PRC2, EZH2 and SUZ12 in a 4E-BP1-dependent manner (Smith et al., 2019). Although the precise mechanism by which mTOR regulates G9a is not yet fully understood, it has been observed that in cells treated with fatty acids, AMPK is suppressed thereby leading to mTORC1 activation, that is paralleled by the increase in levels of G9a (Arjamand Mushtaq et al., 2023).

The mTOR pathway regulates metabolism in response to various stimuli, such as nutrients, energy status or insulin, which result in the production of metabolites that play a role in histone methylation (Laribee, 2018). Among these metabolites, SAM and α-KG have been identified as important regulators of histone methylation (van der Knaap & Verrijzer, 2016). Given that SAM and α-KG are produced through the serine-glycine-one-carbon pathway, the activation of this pathway by mTORC1 through the ATF4-MTHFD2 axis underscores its significance in histone methylation (Ben-Sahra et al., 2016; Cheng et al., 2014; Katrin Düvel et al., 2010). Moreover, the role of mTOR in citric acid cycle (CAC) is critical, and inhibiting mTOR has been found to alter the levels of CAC intermediates, including a-KG, 2HG, succinate, and fumarate (Batsios et al., 2019; Drusian et al., 2018; Morita et al., 2013). These metabolites are known to participate in the demethylation process (van der Knaap & Verrijzer, 2016), suggesting that mTOR may influence histone demethylation by regulating metabolite levels.

Based on the known functions of mTOR in regulating cellular metabolism and epigenetic enzymes, we hypothesized that mTOR may affect cell proliferation by modulating histone methylation. To test this hypothesis, we focused on three well-studied histone methylation marks, viz. H3K4me3 as an active mark, and H3K9me3 and H3K27me3 as repressive marks. Our study aimed to achieve the following three aims:

Aim 1: Investigate the impact of mTORC1 hyperactivation on histone methylation using TSC2-null MEFs, which possess constitutively active mTORC1 (Results presented in section 3.1).

Aim 2: Examine the impact of mTOR inhibition on histone methylation and elucidate the underlying mechanisms involved in regulating histone methylation upon mTOR inhibition (Results presented in sections 3.2-3.5).

Aim 3: Determine the functional role of histone methylation in responses to mTOR inhibitors (Results presented in section 3.6).

CHAPTER 2. Experimental Procedures

1. Cell lines and reagents

TSC2 wild type and knockout mouse embryonic fibroblasts (MEFs) were obtained from Dr. Masahiro Morita. HEK293T was obtained from ATCC. Cells were cultured in Dulbecco modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, and 2 mM l-glutamine (Wisent Bio). The breast cancer cell lines from MMTV-PyMT (mouse mammary tumour virus-polyoma middle tumour-antigen)/4E-BP 1/2-null mice were obtained from Predrag Jovanovic in Dr. Ursini-Siegel's lab. Cells from Jovanovic were cultured in DMEM medium supplemented with 2.5% heat-inactivated fetal bovine serum (FBS), 100 UI/ml penicillin/streptomycin, 50 ug/ml gentamicin and 1% mammary epithelial growth supplement made in house. MCF7 and HCT116 were obtained from ATCC. MCF7 and HCT116 were cultured in RPMI-1640 and DMEM respectively, supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, and 2 mM l-glutamine. Inducible Raptor and Rictor knockout MEFs were provided from Dr. Masahiro Morita (Cybulski et al., 2012). Cells were treated with 5 uM of 4-hydroxytamoxifen (4-OHT) for at least three days. The tumor-derived cell line DIPG13, carrying the H3K27M mutation, and a paired set of DIPG13 H3K27M-KO cells was provided from Dr. Nada Jabado's lab (A. S. Harutyunyan et al., 2019). Cells maintained in Neurocult NS-A proliferation media supplemented with bFGF (10 ng/ml), rhEGF (20 ng/ml), and heparin (0.0002%) on plates coated in poly-L-ornithine (0.01%) and laminin (0.01 mg/ml). For all cells other than DIPG13, 0.05% Trypsin-EDTA was utilized to dislodge the cells from the plate. By contradistinction, accutase was used to detach DIPG13 cells from the plate. Cells were grown in a humidified environment at 37 °C with 5% CO_2. Reagents are listed in Table 1.

2. Lentiviral packaging and infection

The transfection of lentiviral constructs was performed using jetPRIME transfection agent according to the manufacturer's protocol (Polyplus transfection). Briefly, HEK293T cells were co-transfected with 3 μg of target shRNA-containing plasmid (human shEZH1, human shEZH2, mouse shEzh2, and shScramble), 2 μg of psPAX2 packaging plasmid, and 1 μg of pMD2.G plasmid. The media was changed 24 h later and collected 48 h post-transfection. The virus-containing media were filtered through a 0.45 μm filter (ThermoFisher Scientific) and mixed with fresh media at a 1:1 ratio before adding to pre-seeded target cells. To enhance transduction efficiency, 4 μg/ml of polybrene (MilliporeSigma) was added to the cells. The cells were infected in two rounds, with a single infection per day. After 24 h of last infection, selection was performed using 2 μg/ml of puromycin (Bio Basic) to collect cells expressing the desired shRNAs. The list of reagents is described in Table 1. The shRNAs employed in this study were obtained from Sidong Huang's lab, and their detailed information is as follows:

Mouse shEzh2 #1: TRCN0000039040

Mouse shEzh2 #2: TRCN0000304506

Human shEZH1 #1: TRCN0000002441

Human shEZH1 #2: TRCN0000002439

Human shEZH2 #1: TRCN0000040074

Human shEZH2 #2: TRCN0000018365

Non-mammalian shRNA control plasmid DNA (shScramble): SHC002 (MilliporeSigma)

3. Western blotting

Cells were washed with ice-cold PBS twice and lysed with RIPA (1% NP40, 0.1% SDS, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM PMSF, 1 mM DTT, 1X PhosSTOP and 1X proteinase inhibitor cocktail). The lysates were sonicated with a probe sonicator (Fisher Scientific Sonic Dismembrator Model 500) for 4 sec x 2 times at 30% power and clarified at 4 °C (10 min at 16,000 g). Protein concentrations in the supernatants were determined using BCA™ kit (ThermoFisher Scientific). Samples were boiled in 5× Laemmli buffer at 95 °C for 5 min, proteins were separated by SDS-PAGE and transferred using wet mini-transfer system omniBLOT Complete Systems (Cleaver scientific) onto nitrocellulose membranes. In most cases, membranes were blocked in 3% skim milk w/v in TBST buffer (0.1% Tween 20 in 1× TBS) and then incubated with primary antibodies, which were prepared in 3% BSA in TBST at 4 °C. Membranes were washed with TBST (3 × 10 min) and incubated for 1 h with HRP-conjugated secondary antibodies, which were prepared in 5% skim milk/TBST. After washing the membranes with TBST (3 × 15 min), specific protein bands were revealed by chemiluminescence using ECL™ (BioRad) reagent on the Azure 600 (Azure Biosystems). The list of antibodies is described in table 2.

4. Cell proliferation assay

For cell proliferation curves, cells were seeded in 6-well plates and incubated overnight. The media were replaced with treatment media containing 100 nM Ink128, 50 nM rapamycin or DMSO as a negative control. In every 24 h, treatment media were aspirated, and the cells were trypsinized. Complete media were added to stop the trypsinization. Samples were collected, stained with trypan blue to exclude dead cells, and counted using an automated cell counter (Invitrogen). Data was analyzed using Graphpad Prism 9 software.

For the IC_{50} curves, cells were seeded in technical duplicates in 6-well plates and incubated overnight. The media were replaced with treatment media containing Ink128 at increasing concentrations for 72 hours. DMSO served as a control for the treatment. After 72 h, cells were collected and counted using a countess automated cell counter (Invitrogen). IC_{50} curves were plotted, and the numerical values were computed using Graphpad Prism 9 software.

5. LC-MS

SAM and SAH levels at steady state were measured by employing LC-MS/MS at the Metabolomics Core Facility of the Goodman Cancer Research Centre. After treatment of mTOR inhibitors for 48 h, cells were washed in ammonium formate three times, then quenched in cold 50% methanol (v/v) and acetonitrile. Cells were lysed following bead beating at 30 Hz for 2 min. Cellular extracts were partitioned into aqueous and organic layers following dichloromethane treatment and centrifugation. Aqueous supernatants were dried down using a refrigerated speed-vac. Dried samples were subsequently resuspended in 50 µl of water. 5 µl of sample was injected onto an Agilent 6470 Triple Quadrupole (QQQ)-LC-MS/MS for targeted metabolite analysis of SAM and SAH. The liquid chromatography was performed using a 1290 Infinity ultra-performance binary LC system (Agilent Technologies, Santa Clara, CA, USA), with the following parameters: column and autosampler temperatures were 10 °C and 4 °C, respectively; flow rate of 0.6 ml/min with a Intrada Amino Acid column 3 µm, 3.0×150mm (Imtakt Corp, JAPAN). The gradient started at 100% mobile phase B (0.3% formic acid in acetonitrile) with a 3 min gradient to 27% A (100 mM ammonium acetate in 80% water / 20% ACN), followed by a 19.5 min gradient to 100% A. This was followed by a 5.5 min hold time at 100% mobile phase A, a 1 min gradient to 100% mobile phase B, and a subsequent re-equilibration time (7 min)

before subsequent injection. The mass spectrometer was equipped with an electrospray ionization (ESI) source, and the samples were analyzed in positive mode. For each quantitated metabolite, multiple reaction monitoring (MRM) transitions were optimized using standards. Transitions for quantifier and qualifier ions were as follows: SAM (399.1 → 136.1 and 399.1 → 97) and SAH (385.1 → 136 and 385.1 → 250.1). Relative concentrations were determined by comparing the sample area under the curve to external calibration curves prepared in water. No adjustments were made for ion suppression or enhancement. The data were analyzed using MassHunter Quant (Agilent Technologies).

6. GC-MS

After treatment of mTOR inhibitors for 48 h, the plates were quickly placed on ice. Cells were washed three times with chilled isotonic saline solution. Subsequently, 300 μL of 80% methanol pre-chilled to −20 °C was added to the cells. Cells were scraped from the wells and transferred to microcentrifuge tubes pre-chilled to −20 °C. 300 μL more of the 80% methanol was added to the leftover cells in the wells, and then scraped, collected, and pooled with the previously collected 300 μL fraction. The cell suspensions were lysed using a Diagenode Bioruptor sonicator (Diagenode Inc.) at 4 °C. The sonication was performed for 10 m with a 30-sec on-off cycle, using the high-power setting. The process was repeated three times to ensure complete recovery of metabolites. After centrifugation (16,000 g, 4 °C), the cell debris was discarded, and the supernatants were transferred to pre-chilled tubes. Subsequently, the supernatants were dried overnight at 4 °C in a CentriVap cold trap (Labconco) to remove the solvent. Dried pellets were dissolved in 30 μL of pyridine containing methoxyamine-HCl (10 $mg \cdot ml^{-1}$) (MilliporeSigma) using a sonicator and vortex. Samples were incubated for 30 min at 70 °C and then transferred to

GC-MS injection vials containing 70 µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacet-amide (MTBSTFA). Sample mixtures were further incubated at 70 °C for 1 h. For GC–MS analysis, a volume of one microlitre was injected for each sample. GC–MS methods were conducted as previously described (Gravel et al., 2016). Data analyses were executed using Agilent ChemStation and MassHunter software (Agilent). Each metabolite was normalized to the peak intensity of myristic acid-D27, and cell numbers were derived from cells seeded in parallel and identical conditions to those collected for GC-MS steady-state analysis. Data were expressed as fold change relative to vehicle (DMSO)-treated cells.

7. Isolation of RNA and RT-PCR analysis

Total RNA was extracted from cells using Trizol following the manufacturer's instructions. cDNA was made from purified total RNA using SensiFAST™ cDNA Synthesis Kit (#BIO-65053, Bioline) as per manufacturer's protocol. The qPCR was conducted using SensiFAST™ SYBR® Lo-ROX kit (#BIO-98050, Bioline) on the AB7300 machine and analyzed using the 7300 system sds Software (Applied Biosystems); reaction was controlled for the absence of genomic DNA amplification. Each experiment was orchestrated in independent triplicate. Primers were designed using Primer3 (https://primer3.ut.ee/) for human genes. Primers for reactions are outlined as below.

β-actin F	CGGCTACAGCTTCACCACCACG
β-actin R	AGGCTGGAAGAGTGCCTCAGGG
EZH1 F	CACCACATAGTCAGTGCTTCCTG
EZH1 R	AGTCTGACAGCGAGAGTTAGCC
EZH2 F	CCAAGAGAGCCATCCAGACT

EZH2 R	GGGAGGAGGTAGCAGATGTC
KDM6A F	TCAAGGTCTCAGATCCAAAGCT
KDM6A R	GTTCTTCTTTTGTCCGCCCA
KDM6B F	ACCCTCGAAATCCCATCACA
KDM6B R	GCTCTCACAAGGCCAGATCT

8. RNA sequencing and processing

Total RNA was extracted according to the Sigma RNA Extraction Kit (#RTN350-1KT, Sigma) protocol. RNA was sent to SickKids Genome centre for poly(A) RNA library preparation, using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina and sequencing of 50 M 100-bp paired-end reads per replicate on the Illumina NovaSEq 6000 platform. The analysis of RNA-seq was done by Dr. Benjamin Lebeau as described in (Lebeau et al., 2022).

9. ChIP sequencing preparation

To ensure accurate normalization of the ChIP-seq data for H3K27me3, we incorporated a Spike-in technique. This involved adding a small amount of external chromatin to the experimental samples before conducting the ChIP reaction, which allowed us to standardize the signal from the experimental samples to the control signal in the final sequencing data (Egan et al., 2016). The cells were grown to 70-80% confluency before being fixed in 4% formaldehyde for 10 min and stored at -80 °C. The resulting pellets were resuspended in 1 ml of ChIP-buffer, which contained 0.25% NP-40, 0.25% Triton X100, 0.25% Sodium Deoxycholate, 0.005% SDS, 50 nM Tris (pH 8), 100 mM NaCl, 5 mM EDTA, 1X PMSF, 2 mM NaF, and 1X cOmplete protease Inhibitor. The samples were then sonicated using a probe sonicator (Fisher Scientific Sonic

Dismembrator Model 500) with 5 cycles at 20% power, 5 cycles at 25% power, and 5 cycles at 30% power. Each cycle lasted 10 sec, and the samples were kept on ice between each cycle to prevent overheating. The resulting lysates were then spun at high speed in a microcentrifuge for 30 min, and the protein concentration was measured using the BCA assay. The samples were diluted to a protein concentration of 2 mg/ml in ChIP-buffer. Then, 50 µl/ml of Protein G Plus-Agarose Suspension Beads were added to the samples for a 3-h incubation period to preclear them. 2% of the sample was collected as input and stored at -20 °C until DNA purification. For H3K27me3 ChIP, the remaining mixture was supplemented with 20 ng of spike-in chromatin and 2 ug of spike-in antibody. The mixture was then subjected to addition of the specific target antibodies and washed beads. Immunoprecipitation was carried out overnight at 4 °C with the mixture of samples, beads, and primary antibody (see table 1 and 2).

The resulting beads were washed once with Wash1, Wash2, and Wash3 [0.10% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8), with 150/200/500 mM NaCl for Wash1, Wash2, Wash3 respectively], followed by a wash with Wash LiCl [0.25 M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 1 mM EDTA, 10 mM Tris (pH 8)], and two washes with TE buffer [10 mM Tris (pH 8), 1 mM EDTA]. The beads were then resuspended in elution buffer [1% SDS, 0.1 M NaHCO3], and the samples were de-crosslinked overnight at 65 °C. 20 µg of Proteinase K was added for 1 h at 42 °C. DNA was then purified using a BioBasic DNA collection column (#SD5005, BioBasic), and DNA concentration was assessed via the Picogreen assay (#P7589, Invitrogen). ChIPed DNA was sent to SickKids Genome centre for sequencing.

10. ChIP sequencing processing

The analysis of ChIP-seq was executed by Dr. Benjamin Lebeau from Dr. Michael Witcher's lab. Quality control of reads and sequencing was assessed by FastQC (Babraham Bioinformatics). Adapters and low-quality reads were removed by Trimmomatics using the following parameters: ILLUMINACLIP:$Adapters:2:30:10 SLIDINGWINDOW:4:30 LEADING:30 TRAILING:30.

Alignment on hg19 human genome was performed using bowtie2 using "-end-to-end --phred33" parameters, and reads were directly sorted and converted to bam format by samtools. As sequencing was performed on different lanes to increase the depth, related reads on separate lanes were merged together by samtools, and the quality of mapping was checked by samstat. Multiple filtering steps – including removal of low-quality aligned reads and reads aligned to mitochondria chromosome or any chromosome other than chr1-22,X,Y – were performed. Multi-mapped reads were also excluded.

For visualization and some downstream analyses, sorted bam files were first indexed by "picard BuildBamIndex" and then converted to bed files using "bedtools", bamtobed function. DEEPTOOLS package was used for heatmap, coverage, and PCA profiles. BigWig files for IGV peak visualization were generated by DEEPTOOLS, bamCoverage function by excluding black regions, "--ignoreDuplicates, --binSize 1000 --normalizeUsingRPKM --effectiveGenomeSize 2462481010" parameters. For Spiked-In samples, the --scaleFactor was calculated for each sample and added to the code.

Heatmaps, profile plot and tracks were generate using deepTools and samtools (6, 17). Heatmaps and Profile plot were generated using 3kb regions centered around the differential peakset

identified by DiffBind and bigwig from MACS2. Both the computeMatrix and plotHeatmaps were run with default parameter; yMax, zMax and colors were adjusted in each condition to better represent the results.

To visualize ChIP-seq and RNA-seq dot plots and their correlation, dot plots were made by combining the RNA-Seq Log_2FC between stimul. (veh) and mTOR-inhibited conditions and the Log_2FC from DiffBind of any called peak annotated on that gene (+/- 1.5kb) or at the promoter regions, using clusterProfiler. Spearman correlation on the dot plot were performed using the ChIP-Seq Log_2FC and RNA-Seq Log_2FC of every peak colocalization with a gene.

11. Nuclear fractionation

Cytosolic, soluble nuclear and insoluble nuclear proteins were isolated using a commercial kit (#ab219177, Abcam) following the manufacturer's instructions. Briefly, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed with cytosolic extraction buffer. After incubation on ice, nuclei were pelleted by centrifugation at 13,000 g for 1 min at 4 °C, and cytosolic proteins transferred to a clean microfuge tube. Nuclei were lysed using soluble nuclear lysis buffer, sonicated, and incubated on ice for 15 min, vortexing every 5 min. Insoluble material was pelleted by centrifugation at 13,000 g for 10 min at 4 °C, and the soluble nuclear fraction transferred to a clean microfuge tube. Isolated protein fractions were stored at −20 °C. Each buffer was supplemented with 100× protease inhibitor cocktail and DTT supplied with the kit.

12. Flow cytometry for cell cycle and H3K27me3 analysis

The cells were harvested by trypsinization and subsequently washed twice with PBS. To render cells permeable, 70% ethanol was added dropwise while vortexing, and then the cells were stored at -20 °C until needed. After that, the cells were centrifuged at 900 g, washed once with cold PBS, and pelleted at 500 g. Next, the cells were washed with PBSA-T (5% BSA/0.1% Triton-100/PBS) at room temperature. The cells were then incubated with H3K27me3 antibody in PBSA-T for 1 h at room temperature, followed by washing with PBS and incubation with secondary anti-Rat Goat Alexa Fluor 488 and 2 µg/ml DAPI analysis in PBSA-T for 30 min before analysis. A BD Fortessa (BD Biosciences) was used to collect a minimum of 10,000 events. Tables 1 and 2 outline the reagents and antibodies that were used, respectively.

13. Immunofluorescence

MCF7 cells were plated onto #1.5 coverslips of 12–15mm diameter in 24 or 12 well plates. Cells were allowed to recover for 24 h, followed by selected treatments. Coverslips were then washed with PBS twice, fixed with 2% paraformaldehyde/PBS for 20 min at room temperature, washed twice with PBS, and then followed by a 20 min fixation in 0.3% Triton-100/PBS solution. The coverslips underwent two washes with PBS and were then blocked with PBSA-T at room temperature for 1 h. Selected primary antibodies were then incubated for 1 h at room temperature or overnight at 4 °C. Coverslips were washed twice with PBS and incubated with the fluorescent secondary antibody with 2 ug/ml DAPI in PBSA-T for 1 h at room temperature. Coverslips were washed twice in PBS, once in ddH2O and then mounted with Fluoromount-G (Invitrogen). Images were acquired with an LSM800 confocal microscope (Carl Zeiss AG) and analysed as previously described (Findlay et al., 2018). In brief, we measured the mean fluorescence intensity (MFI) of H3K9me3 foci relative to the background signal of the nucleus. Each data

point represents a normalized signal on a per cell basis. Images were analysed using the open-source Java ImageJ/Fiji program. Nuclei were first identified, by thresholding on DAPI fluorescence followed by analysing particles larger than 75 μm^2. H3K9me3 foci were counted using the Find Maxima feature and Measure function. At least 100 cells were analysed per experiment. Tables 1 and 2 outline the reagents and antibodies that were employed, respectively.

14. Micrococcal nuclease (MNase) assay

The following protocol was obtained from Dr. Benjamin Lebeau in Dr. Michael Witcher's lab. MCF7 cells were washed twice with ice-cold PBS and collected by scraping. The cell suspensions were then centrifuged at 300 g at 4° C for 10 min to pellet the cells. After discarding the supernatant, the pellet was washed with 1 ml of ice-cold PBS and spun down again at 300 g at 4 °C for 10 min. The pellet was then resuspended in 1 ml of NP-40 lysis buffer (0.5% NP-40, 10 mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl2, 0.5 mM PMSF, and 1 mM DTT) and incubated for 5 min on ice. The nuclei were pelleted at 120 g at 4 °C for 10 min, and the supernatant was discarded. The nuclei were then resuspended in HeLa dialysis buffer (HDB) with $CaCl_2$ (20 mM HEPES pH 7.5, 50 mM KCl, 10% glycerol, 1 mM $CaCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM DTT), and 10 µl of input was collected. Next, 10 U of MNase was added to 100 µl of resuspended nuclei, and the mixture was incubated for 2, 5, 15, and 60 min. After each time point, 10 µl of sample was taken in a new tube containing 1 µl of 0.1 M EDTA to stop the MNase activity. To the mixture, 89 µl of HDB with EDTA (20 mM HEPES pH 7.5, 50 mM KCl, 10% glycerol, 0.01 M EDTA, 0.5 mM PMSF, and 1 mM DTT) was added to bring the volume up to 100 µl. Then, 90 µl of WSN buffer (30 µl of water, 20 µl of 10% SDS, and 40 µl of 5 M NaCl) and 5 µl of 20 µg/µl RNase were added, and the mixture was incubated at 37 °C for 20

min. After incubation, 5 µl of 20 µg/µl Proteinase K was added, and the mixture was incubated at 65 °C for 1 h. To extract DNA, PB buffer was added to the mixture and transferred to a column (#SD5005, BioBasic). After centrifugation at 13,000 rpm for 1 min, the flowthrough was discarded, and PE buffer was added. After centrifugation at 13,000 rpm for 1 min, the column was moved to a clean tube. The DNA was eluted with 30 µl of nuclease-free water by centrifuging at 13,000 rpm for 1 min. Finally, the eluted DNA was analyzed on a 1% agarose gel with RedSafe and detected using the Azure 600 instrument (Azure Biosystems). Table 1 outlines the reagents that were used.

15. Immunoprecipitation

The cells were grown in a 15cm cell culture dish and washed twice with ice-cold PBS before being scraped. The cells were then lysed using lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, 0.5 mM DTT, 1 mM PMSF and 1x EDTA-free cOmplete protease inhibitor cocktail (Roche Applied Science). The protein concentration was determined via BCA assay, and 1 mg of protein was diluted in lysis buffer. Protein G agarose beads were washed with lysis buffer. To pre-clear the lysates, they were incubated with protein G agarose beads, followed by centrifugation to pellet the beads. Immunoprecipitation was performed using anti-EZH2 rabbit polyclonal antibody or rabbit IgG as a control, according to the manufacturer's protocol, with 1-h incubation. The washed beads were then added to the tube and incubated for 1 h with rotation. The mixture was washed three times with the buffer, and the beads were resuspended in 2X SDS loading buffer. Total cell lysates and immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting. Tables 1 and 2 outline the reagents and antibodies that were employed, respectively.

Table 1. The list of reagents

Name	Cat.	Company
16% Formaldehyde Solution	28908	Thermofisher
4-hydroxytamoxifen	H7904	MilliporeSigma
Accutase	A6964	MilliporeSigma
alpha-ketoglutarate (a-KG)	75890	MilliporeSigma
bFGF, ACF	78134.1	StemCell Technologies
cOmplete protease inhibitor	04693116001	Roche
cOmplete, EDTA-free	11873580001	Roche
DAPI	D9542	MilliporeSigma
Dimethyl ketoglutarate (DMKG)	349631-5G	MilliporeSigma
DMEM	319-005-CL	Wisent
DMSO	67-68-5	BioBasic
Fluoromount-G™ Mounting Medium	0-4958-02	Invitrogen
Gentamycin sulfate	450-135-XL	Wisent
GSK126	HY-13470	MedChemExpress
GSKJ4	SML0701	MilliporeSigma
Heparin 0.2%	07980	StemCell Technologies
Ink128	S2811	Selleckchem
L-Glutamine	609-065-EL	Wisent
Laminin 100X (EHS murine sarcoma)	L2020	MilliporeSigma
Neurocult NS-A Basal Medium kit (human)	05751	StemCell Technologies

Paraformaldehyde	P6148	Thermofisher
PB buffer	19066	Qiagen
PBS	311-425-CL	Wisent
PE buffer	19065	Qiagen
PhosphoSTOP	04906837001	Roche
Poly-L-ornithine 0.01%	P4957	MilliporeSigma
Polybrene	107689	MilliporeSigma
Protein G Plus-Agarose Suspension	IP04-1.5ML	MilliporeSigma
Proteinase K	39450-01-6	Sigma
Rapamycin	1292	Tocris Bioscience
RedSafe	21141	FroggaBio
rhEGF	78006	StemCell Technologies
RPMI1640	350-000-CL	Wisent
Trizol	15596018	Ambion
UNC0642	HY-13980	MedChemExpress
UNC1999	HY-15646	MedChemExpress

Table 2. The list of antibodies

Antibody	Cat.	Company
4E-BP1	9644	Cell Signaling Technologies
AKT	4691	Cell Signaling Technologies
Amersham ECL Mouse IgG, HRP-linked whole Ab (from sheep)	NA931	Cytiva
Anti-IgG Donkey Polyclonal Antibody (HRP (Horseradish Peroxidase))	95017-556L	Cytiva
Anti-Rat Goat Alexa Fluor 488	A-11070	ThermoFisher Scientific
b-actin	A1978	MilliporeSigma
EED	ab4469	Abcam
EZH1	42088	Cell Signaling Technologies
EZH2	5246	Cell Signaling Technologies
G9a (EHMT2)	3306	Cell Signaling Technologies
GLP (EHMT1)	35005	Cell Signaling Technologies
H2A	ab18255	Abcam
H2AK119Ub	8240	Cell Signaling Technologies
H3	ab1791	Abcam
H3K27me2	9728	Cell Signaling Technologies
H3K27me3	9733	Cell Signaling Technologies
H3K4me3	07-473	MilliporeSigma
H3K9me2	4658	Cell Signaling Technologies

H3K9me3	39161	Active Motif
KDM6A	33510	Cell Signaling Technologies
KDM6B	PA5-72751	ThermoFisher Scientific
Normal Rabbit IgG	10500C	Cell Signaling Technologies
p-4E-BP1 (S65)	9456	Cell Signaling Technologies
p-AKT (S473)	4060	Cell Signaling Technologies
p-rpS6 (S240/244)	2215	Cell Signaling Technologies
Raptor	09-217	MilliporeSigma
RBBP4	9067	Cell Signaling Technologies
Rictor	2114	Cell Signaling Technologies
rpS6	sc-74459	Santa Cruz Biotechnologies
Spike-in Antibody	61686	Active Motif
Spike-in Chromatin	53083	Active Motif
SUZ12	3737	Cell Signaling Technologies
TSC2	4308	Cell Signaling Technologies
α-tubulin	T5168	MilliporeSigma

CHAPTER 3. Results

1. Investigating the impact of mTORC1 hyperactivation on histone methylation using TSC2-null MEFs, which possess constitutively active mTORC1.

1.1. Selective induction of H3K27me3 is observed upon constitutive mTORC1 activation.

In our investigation into the role of mTOR activation on histone methylation, we utilized TSC2-null MEFs as a model for constitutive activation of mTORC1 signaling (Hongbing Zhang et al., 2003). TSC2 is known to possess GAP activity towards RHEB, a small GTPase from the Ras family, and TSC2 acts as an antagonist of the mTOR signaling pathway by facilitating GTP hydrolysis of RHEB (Inoki et al., 2002). As expected, we observed increased mTORC1 activity in TSC2-null cells, as indicated by elevated levels of phosphorylated rpS6 (S240/244) and 4E-BP1 (S65), as compared to wile-type (WT) cells (Fig 2.1A). To investigate the effect of TSC2 on histone methylation, we compared the commonly studied histone methylation marks, namely H3K4me3, H3K9me3, and H3K27me3 between TSC2 WT and KO cells. Our results showed that TSC2-null cells exhibited a specific increase in H3K27me3, a type of histone methylation known to be linked to gene suppression, as compared to WT cells. In contrast, the levels of other histone methylation marks such as H3K4me3 and H3K9me3 were largely unaffected by the TSC2 status in the cell. (Fig 2.1A)

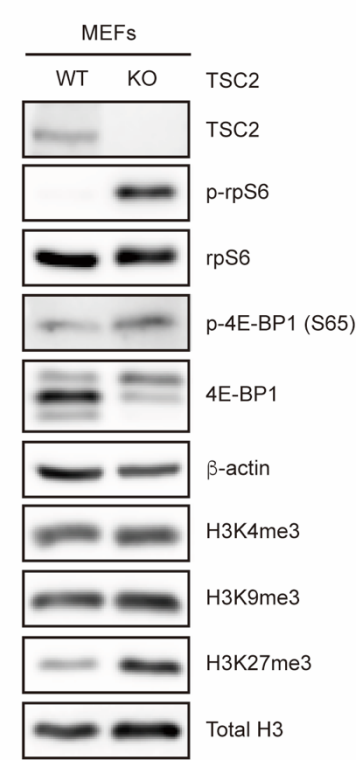


Fig 2.1. Constitutively active mTORC1 via TSC2 KO induces H3K27me3 selectively

A. Levels of the indicated proteins in TSC2 WT and KO MEFs were determined by Western blotting. β-actin served as a loading control.

1.2. Constitutive mTORC1 activation elevates H3K27me3 through the 4E-BP1/EZH2 axis.

Given that mTORC1 is known to promote EZH2 protein synthesis (Smith et al., 2019), we investigated the EZH2 protein level and found that TSC2-null cells exhibited the higher level of EZH2 than WT cells (Fig 2.2A). To examine whether the increase in EZH2 was mediated by the mTORC1/4E-BP1 axis as reported (Smith et al., 2019), we used breast cancer cells from MMTV-PyMT/Eif4ebp1/2-null mice. We introduced into these cells either of two types of genetic constructs, viz. empty vector (EV) or human 4E-BP1, thereby gauging the impact of 4E-BP1 on EZH2 and H3K27me3. Our results showed that cells rescued by 4E-BP1 displayed reduced levels of H3K27me3 compared to cells lacking 4E-BP1/2 (Fig 2.2B). This change in H3K27me3 was accompanied by corresponding changes in EZH2 expression. (Fig 2.2B). In conclusion, these results confirm that mTORC1 increases EZH2 protein synthesis by phosphorylating and inactivating 4E-BPs.

To further determine whether EZH2 is the primary regulator of H3K27me3 induction resulting from mTORC1 hyperactivation, we employed shRNAs to specifically suppress Ezh2 expression in both TSC2 WT and KO cells. Our findings demonstrated that Ezh2 knockdown successfully reduced EZH2 levels in both TSC2 WT and KO cells, which was associated with a decrease in H3K27me3 levels (Fig 2.2C). Additionally, we observed that the elevation of H3K27me3 displayed in TSC2 KO cells was abolished upon knockdown of Ezh2 (Fig 2.2C). We also conducted experiments to examine the impact of pharmacological inhibition of EZH2 using GSK126, an EZH2 inhibitor, on H3K27me3 levels. Our results showed that treating the cells with GSK126 led to a reduction in H3K27me3 levels in a dose-dependent manner (Fig 2.2D). Importantly, when TSC2-null cells were treated with concentrations of GSK126 higher than 2 uM, they did not display higher levels of H3K27me3 compared to WT cells (Fig 2.2D), which

suggests that EZH2 is the main regulator of H3K27me3 induction resulting from mTORC1 hyperactivation.

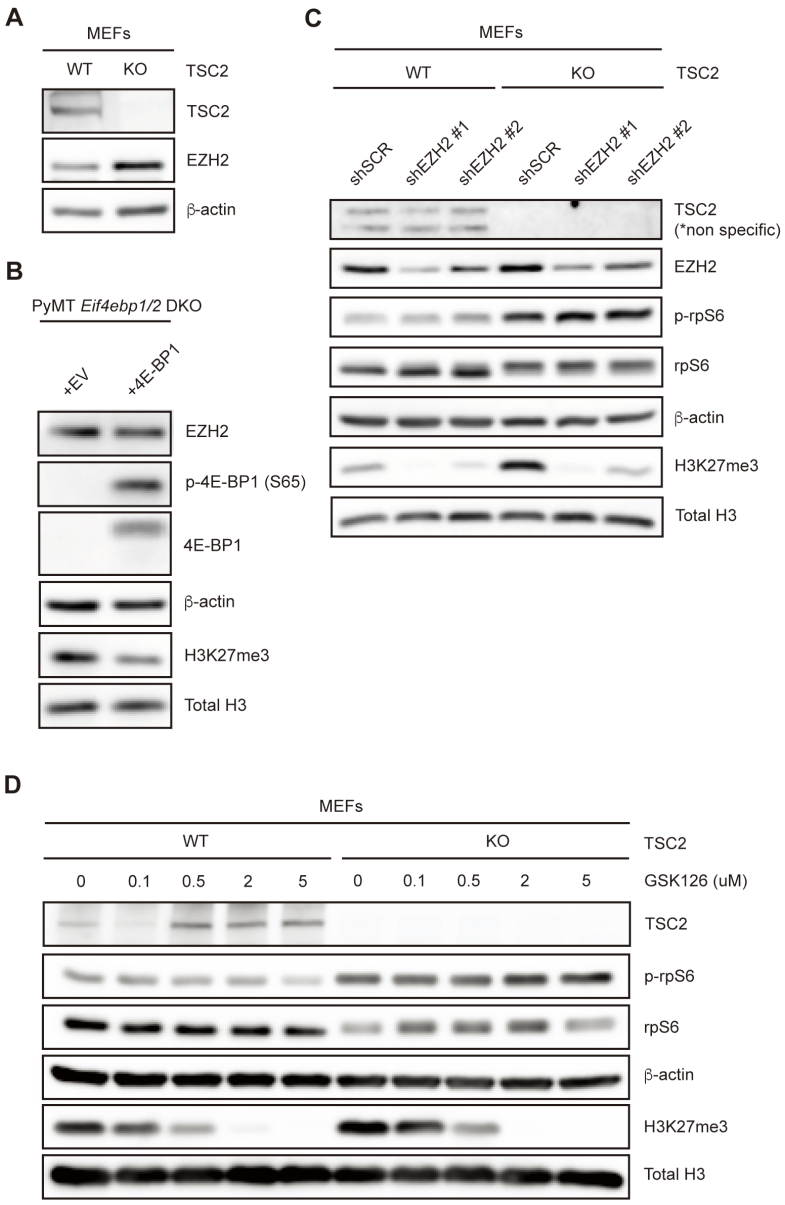


Fig 2.2. Constitutive mTORC1 activation elevates H3K27me3 through the 4E-BP1/EZH2 axis

(A) Levels of the indicated proteins in TSC2 WT and KO MEFs were determined by Western blotting. β-actin served as a loading control. (B) Levels of the indicated proteins in Eif4ebp1/2 DKO cells infected with EV and human 4E-BP1 were assessed by Western blotting. β-actin served as a loading control. (C) Levels of the indicated proteins in TSC2 WT and KO MEFs

infected with a scrambled (shSCR) or Ezh2-specific shRNA (shEzh2) were determined by Western blotting. β-actin served as a loading control. (D) Levels of the indicated proteins in TSC2 WT and KO MEFs treated with the EZH2 inhibitor (GSK126) in different doses for 48 h were determined by Western blotting. β-actin served as a loading control.

2. mTOR inhibition induces hypermethylation of H3K27

2.1. mTOR inhibition induces an increase in H3K27me3 levels independent of TSC2

Since mutations in upstream regulators such as PI3KCA, PTEN, and TSC2 often result in high mTOR activity in cancer, the mTOR pathway is being explored as a possible target for anti-tumour therapy (Ho et al., 2017; Li et al., 1997; Liu et al., 2009; Mehta et al., 2011; Samuels & Waldman, 2010). Therefore, our study aimed to investigate the effects of mTOR inhibitors in TSC2 WT and KO MEFs on histone methylation. Following overnight serum starvation, we treated cells with Ink128, a commonly used asTORi, in the presence of 10% FBS for durations of 4, 24 and 48 h. Our findings confirmed higher mTORC1 activity in TSC2-null cells and demonstrated a decrease in mTOR activity, as indicated by reduced levels of p-rpS6 and p-4E-BP1 at all time points (Fig 2.3A) following Ink128 treatment. H3K27me3 levels were unaffected by acute mTOR inhibition (4 h), but increased with prolonged mTOR inhibition (24h and 48 h) in both TSC2 WT and KO cells (Fig 2.3A). Importantly, the elevation of H3K27me3 levels occurred independently of TSC2 status in the cell (Fig 2.3A). Furthermore, we examined the expression of EZH2, which was initially higher in TSC2-null cells compared to WT cells but decreased after 24 h of Ink128 treatment (Fig 2.3A), which is likely due to regulation through the mTORC1/4E-BP1 axis. These findings suggest that the mechanism by which mTOR inhibition influences H3K27me3 may be distinct from mTORC1 activation. Consequently, we conducted further investigations to explore the impact of mTOR inhibition on histone methylation.

To examine the impact of mTOR inhibition on histone methylation, we utilized three methods of mTOR inhibition in cancer cells (MCF7-breast cancer and HCT116-colorectal cancer) harbouring PI3K mutations. These methods included the use of an allosteric mTOR inhibitor

(rapamycin), an asTORi (Ink128), or serum starvation, which restricts growth factors and leads to mTOR inhibition (Pirkmajer & Chibalin, 2011). As previously described, cells were treated with mTOR inhibitors or serum starvation with 10 % FBS after overnight serum starvation. The efficient inhibition of mTOR was presented by decreased phosphorylation of rpS6 and/or 4E-BP1 at both 24 h and 48 h time points in both cell lines (Fig 2.3B-C). In addition, our results validated the hypotheses that rapamycin treatment indeed induces AKT signaling through phosphorylation at the S473 site of AKT (Wan et al., 2007) and that inhibition of 4E-BP1 phosphorylation by rapamycin is not as effective as Ink128, as previously reported (Choo et al., 2008) (Fig 2.3B-C). Upon assessing the efficacy of mTOR signaling inhibition, we subsequently examined the impact on histone methylation marks. Our findings revealed that mTOR inhibition resulted in increased levels of H3K9me3 and H3K27me3, two repressive histone methylation marks, while H3K4me3 remained unchanged in MCF7 (Fig 2.3B). Unlike MCF7, mTOR inhibition resulted in a slight induction of H3K27me3 in HCT116 cells, but its impact on histone methylation was subtle (Fig 2.3C). Therefore, we chose MCF7 as our primary cell model for further research because we can observe a similar effect of mTOR inhibition on H3K27me3 as in MEFs.

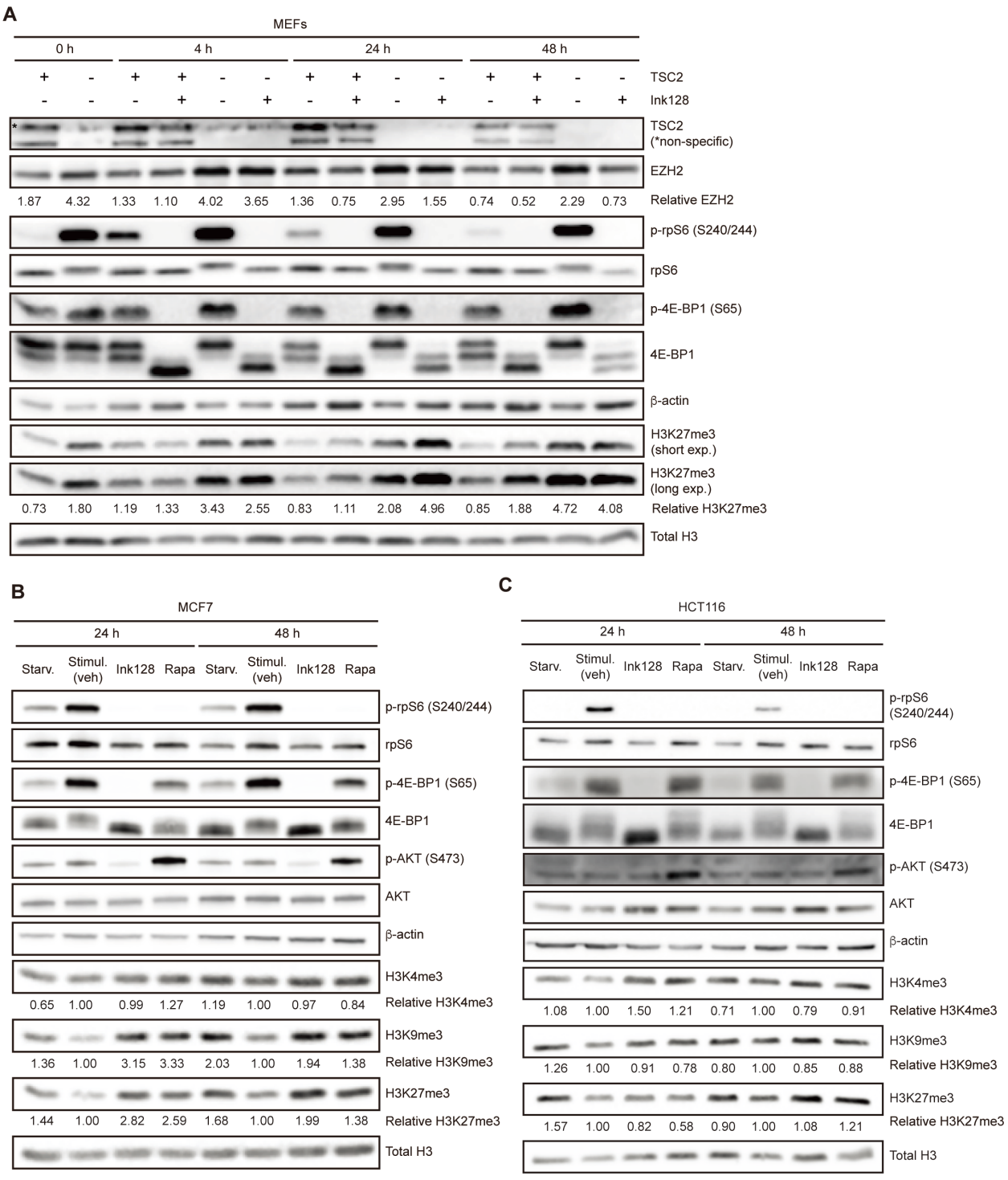


Fig 2.3. mTOR inhibition elevates H3K9me3 and H3K27me3, but not H3K4me3

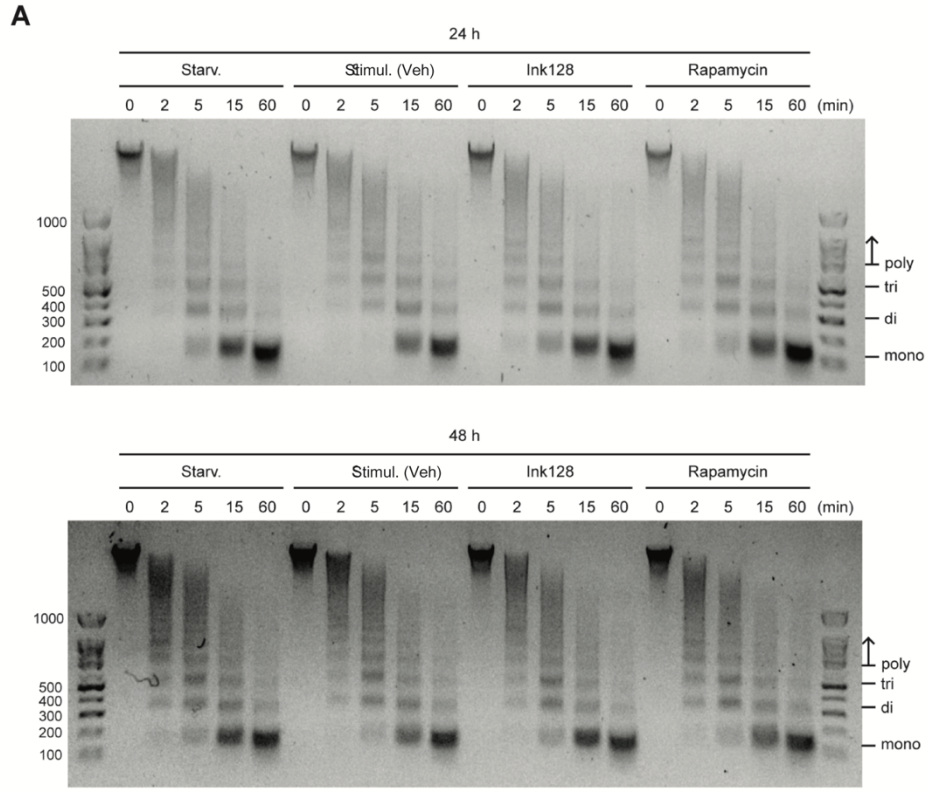
(A) TSC2 WT and KO MEFs were serum-starved overnight and then stimulated with 10% FBS in the presence of Ink128 (100 nM) for the indicated periods. Levels of the indicated proteins

were monitored by Western blotting. The intensity of EZH2 and H3K27me3 was quantified by ImageJ and normalized to the intensity of β-actin and H3, respectively. (B-C) MCF7 (B) and HCT116 (C) cells were serum-starved overnight and then stimulated with 10% FBS in the presence of Ink128 (100 nM) or rapamycin (50 nM) for the indicated periods. Serum starvation was employed as a method to inhibit mTOR. Levels of the indicated proteins were monitored by Western blotting. The relative intensity of histone methylation marks was quantified by ImageJ and normalized to the intensity of H3.

2.2. mTOR inhibition is not paralleled by significant alterations in H3K9me3 levels.

H3K9me3 is considered to be a marker for heterochromatin that recruits other epigenetic modifications to maintain closed chromatin structure (Becker et al., 2016), thus rendering this histone modification important for assessing the role of mTOR in chromatin compaction. To confirm whether mTOR inhibition regulates chromatin formation, we utilized the MNase assay, a commonly used technique for studying nucleosome occupancy and assessing chromatin accessibility (Tsompana & Buck, 2014) to determine the formation of mono/di-nucleosomes in response to mTOR inhibition. These experiments showed that there was no significant change in mono/di-nucleosome formation following mTOR inhibition, indicating that mTOR inhibition does not alter chromatin accessibility in our model (Fig 2.4A). Given the absence of any discernible changes in chromatin compaction subsequent to mTOR inhibition, we proceeded to examine the impact of mTOR inhibition on H3K9me3 using alternative approaches, including immunofluorescence (IF) and ChIP-seq. Considering that H3K9me3 is typically situated at the nuclear periphery, albeit with a few focal points in the centre of the nucleus (Ugarte et al., 2015) (Fig 2.4B), we employed IF to quantify the number of H3K9me3 foci and measure the mean fluorescence intensity (MFI). We observed that Ink128 treatment did not affect the number of H3K9me3 foci, whereas serum starvation and rapamycin treatment led to a slight decrease in the foci number (Fig 2.4C). Furthermore, the MFI of H3K9me3 normalized to DAPI intensity was lower in mTOR-inhibited cells compared to the control cells (Fig 2.4D). Additionally, we conducted ChIP-seq to examine the enrichment of H3K9me3 in response to mTOR inhibition, and generated a profile plot of H3K9me3 in each treatment. Our analysis showed that mTOR inhibition did not lead to a substantial change in H3K9me3 levels (Fig 2.4E). Overall, in our model, the inhibition of mTOR did not exhibit a significant effect on chromatin accessibility, and

the changes in H3K9me3 following mTOR inhibition were observed differently with various detection methods. This suggests that a more suitable detection method should be employed to draw conclusions regarding the effect of mTOR inhibition on H3K9me3.


A
24 h
Starv.
Stimul. (Veh)
Ink128
Rapamycin
0 2 5 15 60 0 2 5 15 60 0 2 5 15 60 0 2 5 15 60 (min)
1000
500
400
300
200
100
poly
tri
di
mono
48 h
Starv.
Stimul. (Veh)
Ink128
Rapamycin
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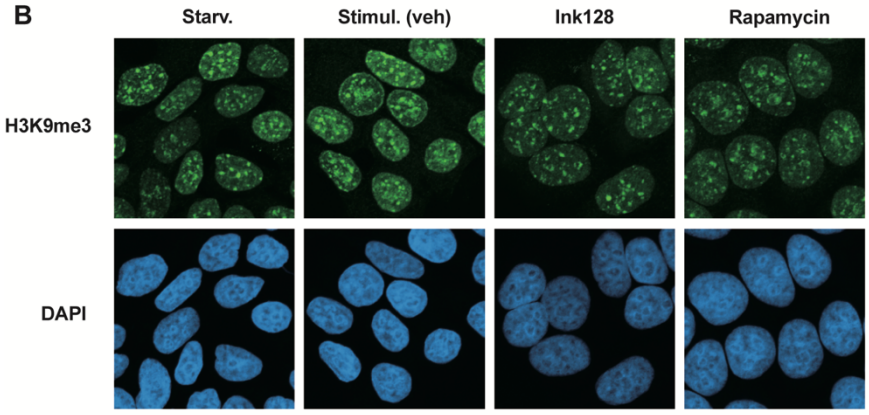
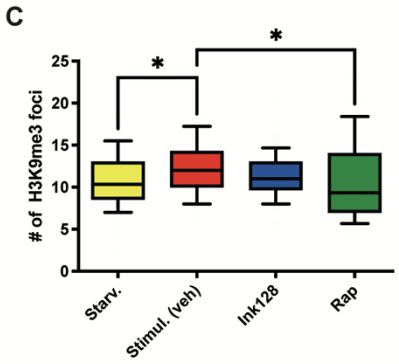
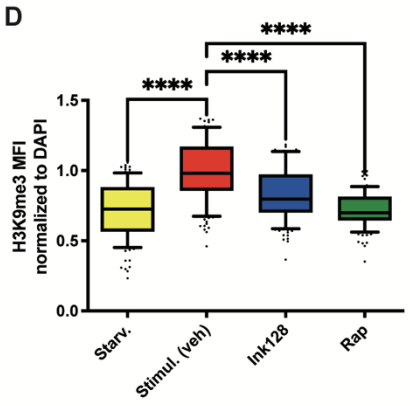

B
Starv.
Stimul. (veh)
Ink128
Rapamycin
H3K9me3
DAPI


C
# of H3K9me3 foci
25
20
15
10
5
0
*
*
Starv.
Stimul. (veh)
Ink128
Rap


D
H3K9me3 MFI normalized to DAPI
1.5
1.0
0.5
0.0
****
****
****
Starv.
Stimul. (veh)
Ink128
Rap

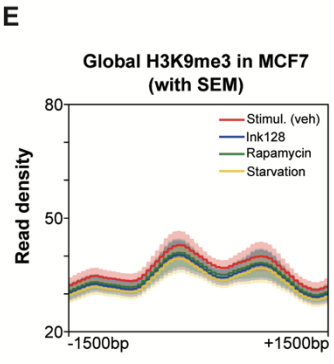

E
Global H3K9me3 in MCF7
(with SEM)
Read density
80
50
20
Stimul. (veh)
Ink128
Rapamycin
Starvation
-1500bp
+1500bp

Fig 2.4. No discernible change in chromatin accessibility or H3K9me3 levels following mTOR inhibition

(A) MCF7 cells were exposed to mTOR inhibitors or subjected to serum starvation for either 24 or 48 h. Following treatment, the nuclei were digested with MNase for the specified time intervals, and the genomic DNA was extracted and resolved on a 1.2% agarose gel. (B) Representative confocal images of IF staining for H3K9me3 (green) and for DAPI (blue) in MCF7 cells following mTOR inhibition for 48 h. Cells were treated, fixed, stained, and imaged via confocal microscopy. (C-D) Quantification of H3K9me3 foci (C) and the mean fluorescence intensity (MFI) (D). Bars represent mean ± SD. *P< 0.05, ****P< 0.0001, one-way ANOVA with Dunnett's post-test compared to Stimul. (veh). (E) The normalized average read peak density profiles (+/− 1.5kb) for H3K9me3 in MCF7.

2.3. ChIP-seq analysis confirmed the induction of H3K27me3, which was found to involve both mTORC1 and mTORC2.

Since upon mTOR inhibition we observed consistent alterations in H3K27me3, but not H3K4me3 or H3K9me3, we shifted our focus to H3K27me3. We firstly confirmed the impact of mTOR inhibition on H3K27me3 enrichment by performing ChIP-seq. According to the profile plot of H3K27me3, mTOR inhibition led to a greater enrichment of H3K27me3 compared to the control group (Fig 2.5A-B), which is in line with our previous findings (Fig 2.3B-C). In parallel with ChIP-seq, RNA-seq was conducted to assess the impact of H3K27me3 alteration on the transcriptome. Given that H3K27me3 is a repressive mark, we observed a negative correlation between the two datasets; however, the correlation was found to be very weak (Fig 2.5C-D). This may indicate that the effect of H3K27 hypermethylation following mTOR inhibition on gene expression is subtle.

In order to gain a deeper understanding of the involvement of mTORC1 and mTORC2 in the regulation of H3K27me3, we utilized inducible knockout MEFs deficient in either the mTORC1-specific component Raptor (iRapKO) or the mTORC2-specific component Rictor (iRicKO) obtained from Dr. Morita (Cybulski et al., 2012). Generating stable knockdown cells of Raptor or Rictor is challenging due to each specific component's essential role in cell proliferation (Saxton & Sabatini, 2017). Complete knockdown of Raptor or Rictor results in slow cell proliferation, and prolonged culture can lead to the emergence of inefficiently "knocked down" cells (Fish & Kruithof, 2004). To overcome this, we utilized an inducible knockout system to investigate the effect of Raptor and Rictor.

To induce Raptor or Rictor knockout, cells were treated with 4-Hydroxytamoxifen (4-OHT) for at least three days, and then serum-starved for 6 hours and stimulated by FBS for 30 min to

confirm mTOR signaling. While we were not able to achieve complete knockout of Raptor or Rictor, we did observe a reduction in Raptor, p-rpS6, and p-4E-BP1 in Raptor knockdown cells (Fig 2.5E) and a decrease in Rictor and p-AKT S473 levels in Rictor knockdown cells, confirming successful knockdown (Fig 2.5F). We also found that both iRapKO and iRicKO cells showed increased levels of H3K27me3 compared to control cells (Fig 2.5E-F). This suggests that ablation of both mTORC1 and mTORC2 leads to an elevation of H3K27me3. Collectively, the induction of global H3K27me3 levels upon mTOR inhibition was confirmed through ChIP-seq analysis, and both mTORC1 and mTORC2 are involved in H3K27me3 alteration.

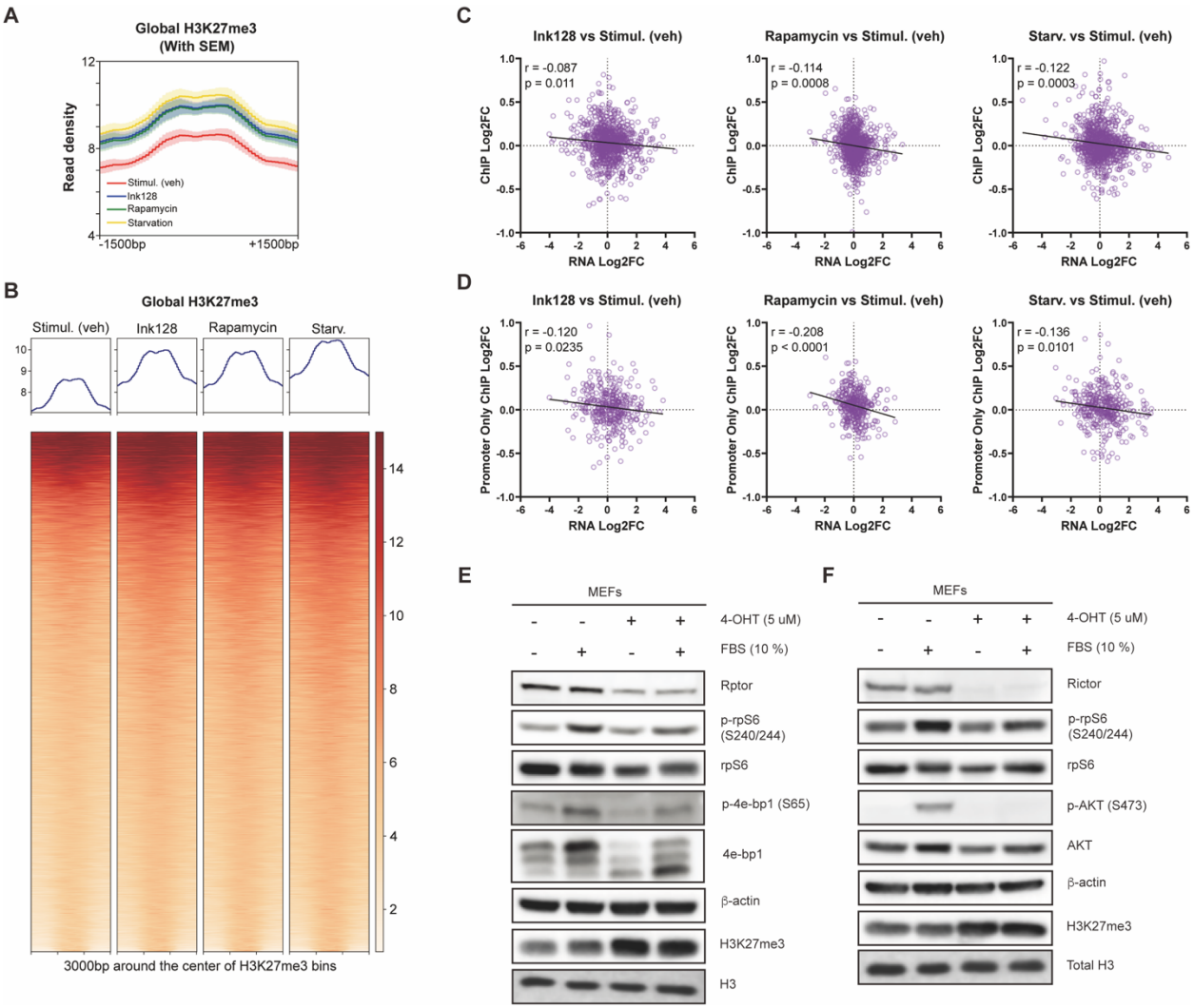


Fig 2.5. ChIP-seq analysis confirms the increase of global H3K27me3 enrichment following mTOR inhibition, shown to be mediated by both mTORC1 and mTORC2

(A-B) The normalized average read peak density profiles for H3K27me3 with SEM (A) and heatmap plots of ChIP-seq signal intensity for H3K27me3 (B) in MCF7 treated as indicated. (C-D) Correlation between ChIP-seq and RNA-seq data across all regions (C) and specifically in the promoter region (D). (E-F) Levels of the indicated proteins in iRapKO (E) and iRicKO MEFs (F) were determined by Western blotting. β-actin was used as a loading control.

3. The alteration in H3K27me3 is mediated by H3K27me3 demethylases or PRC2.

3.1. The induction of H3K27me3 upon mTOR inhibition cannot be attributed to changes in the cell cycle.

In light of the facts that new histone modifications are incorporated into chromatin during cell division (Ma et al., 2015) and that mTOR inhibition results in G1/S arrest (Dowling et al., 2010), we conducted an investigation to determine whether the observed increase in H3K27me3 upon mTOR inhibition may be mediated by cell cycle progression. To examine this, we treated cells with either mTORi or serum starvation, and used DAPI staining to detect DNA content, as well as an antibody probe for H3K27me3. Using flow cytometry, we observed that both serum starvation and mTORi treatment induced G1/S arrest as reported (Fig 2.6A) (Dowling et al., 2010). Additionally, we observed increased levels of H3K27me3 following mTOR inhibition (Fig 2.6B), which is consistent with our previous findings. We then evaluated the intensity of H3K27me3 in each phase of the cell cycle and discovered that H3K27me3 did not significantly change between the different cell cycle phases (Fig 2.6C). This indicates that the changes observed in H3K27me3 are not caused by the effects of mTOR inhibition on the cell cycle.

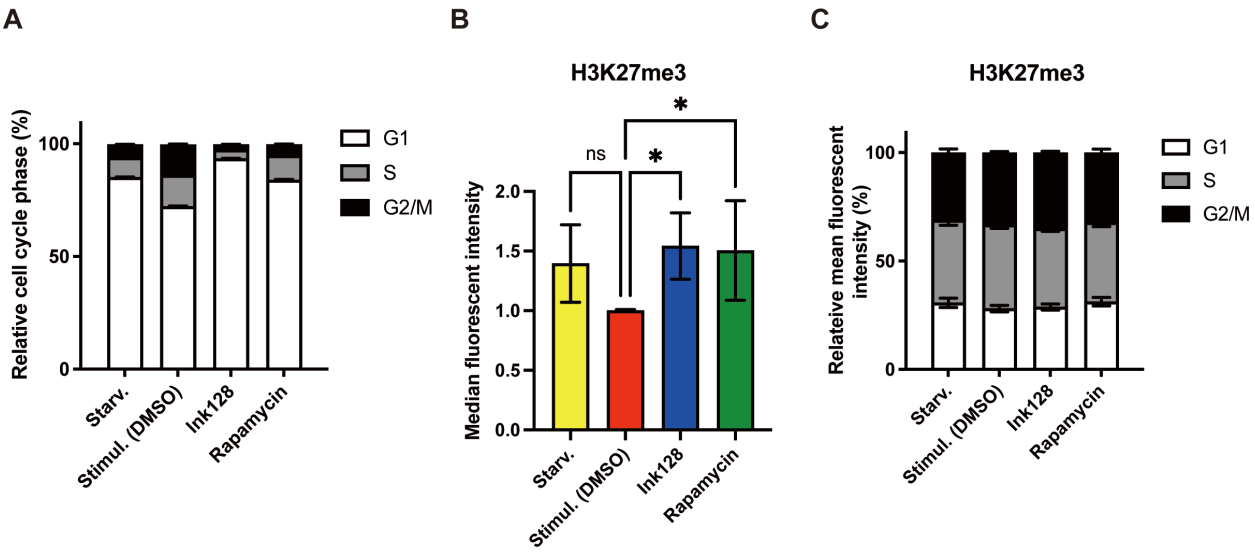


Fig 2.6. The alteration of H3K27me3 is not mediated by the cell cycle changes following mTOR inhibition.

(A) Flow cytometric cell cycle profile of MCF7 cells upon mTOR inhibition for 48 hours. (B) The overall mean fluorescent intensity of H3K27me3 was detected by flow cytometry. ns: no significancy, *P< 0.05, one-way ANOVA with Dunnett's post-test compared to Stimul. (DMSO). (C) The mean fluorescent intensity of H3K27me3 in each cell phase. Bars represent mean ± SD.

3.2. The hypermethylation of H3K27 in response to mTOR inhibition is solely attributed to the involvement of H3K27 demethylases or PRC2.

H3K27 methylation can be regulated by writers and erasers. KDM6A and KDM6B are primary demethylases of H3K27me3 (Swigut & Wysocka, 2007), while EZH2 is the catalytic subunit of PRC2 that mediates H3K27me3 deposition (Guo et al., 2021). Prior to a detailed investigation, we aimed to determine if these two mechanisms are exclusively responsible for H3K27 hypermethylation following mTOR inhibition. To validate this hypothesis, we employed GSK126 as an EZH2 inhibitor and GSKJ4 as a KDM6A/B inhibitor. As depicted in Figure 2.7, we observed a significant decrease in H3K27me3 levels upon treatment with GSK126 and an increase in H3K27me3 levels upon treatment with GSKJ4 in cells treated with the vehicle (DMSO). Interestingly, even in the presence of mTOR inhibitors, GSKJ4 treatment resulted in a slight induction of H3K27me3 (Fig 2.7). Furthermore, GSK126 effectively countered the impact of mTOR inhibition on H3K27me3 by reducing its levels (Fig 2.7). Upon simultaneous inhibition of both demethylases and EZH2, we did not observe notable alterations in H3K27me3 levels following mTOR inhibition (Fig 2.7). These findings suggest that PRC2 or demethylases exclusively contribute to H3K27 hypermethylation in response to mTOR inhibition.

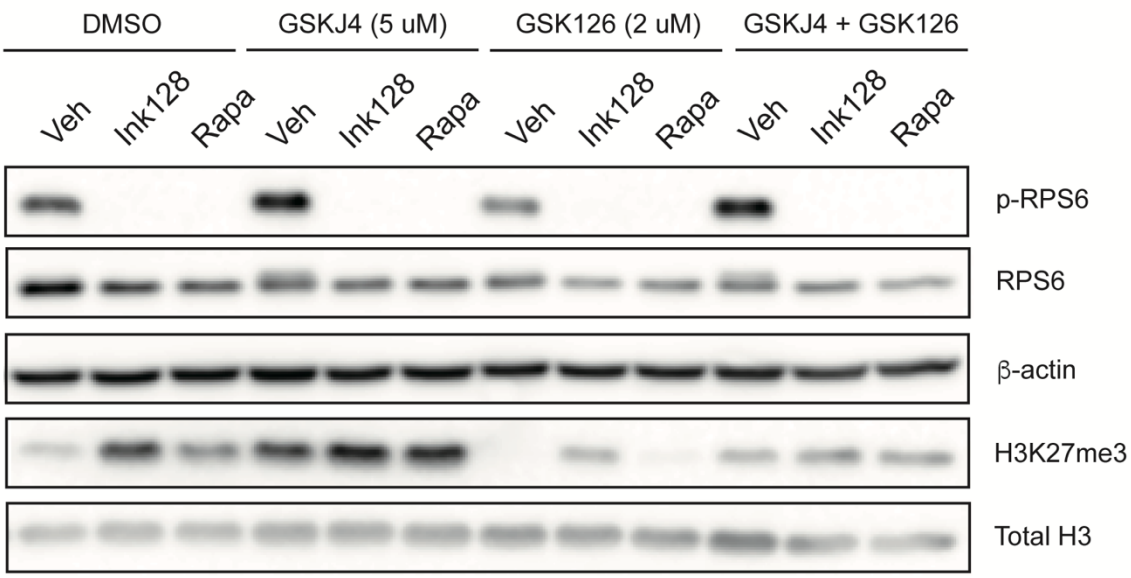


Fig 2.7. The increase in H3K27me3 following mTOR inhibition is exclusively caused by the involvement of PRC2 or demethylases

MCF7 cells were treated with GSKJ4, GSK126, or both, in the presence of mTOR inhibitors for 48 h. Phosphorylation and expression levels of indicated proteins were monitored by Western blotting. β-actin served as a loading control.

4. The H3K27 hypermethylation upon mTOR inhibition occurs independently of demethylation processes.

4.1. Changes in the levels and localization of KDM6A and KDM6B cannot explain mTORi-induced changes in H3K27me3.

As previously stated, upon mTOR inhibition, the induction of H3K27me3 can be achieved exclusively through the involvement of demethylases or PRC2. Initially, we assessed the expression levels and protein abundance of KDM6A and KDM6B. The expression levels of both demethylases were elevated upon mTOR inhibition (Fig 2.8A). Furthermore, in comparison to the control, only the KDM6A protein level exhibited a slight increase with Ink128 treatment, while the protein level of KDM6B remained unaltered (Fig 2.8B). This suggests that the observed H3K27 hypermethylation following mTOR inhibition cannot be attributed to a change in KDM6A/6B protein levels. We then investigated the localization of KDM6A/B, since the activity of epigenetic modifiers is influenced by localization (Feinberg et al., 2016). Interestingly, we found that mTOR inhibition did not affect the localization of either KDM6A or KDM6B (Fig 2.8C). Collectively, these findings imply that the changes in the levels/localization of KDM6A and KDM6B cannot account for the alterations in H3K27me3 induced by mTOR inhibitors.

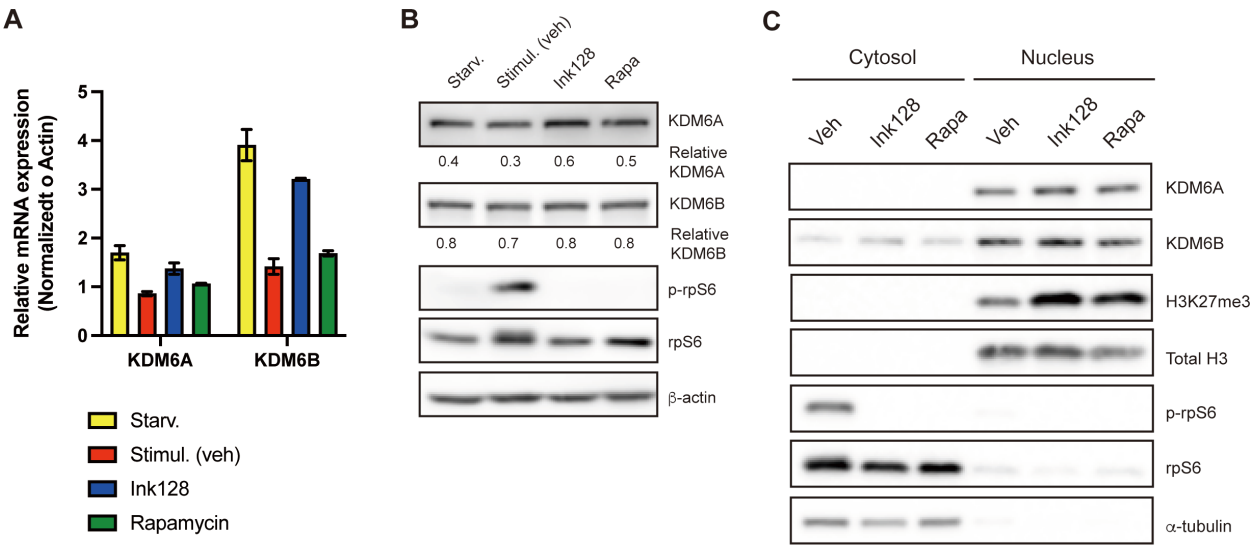


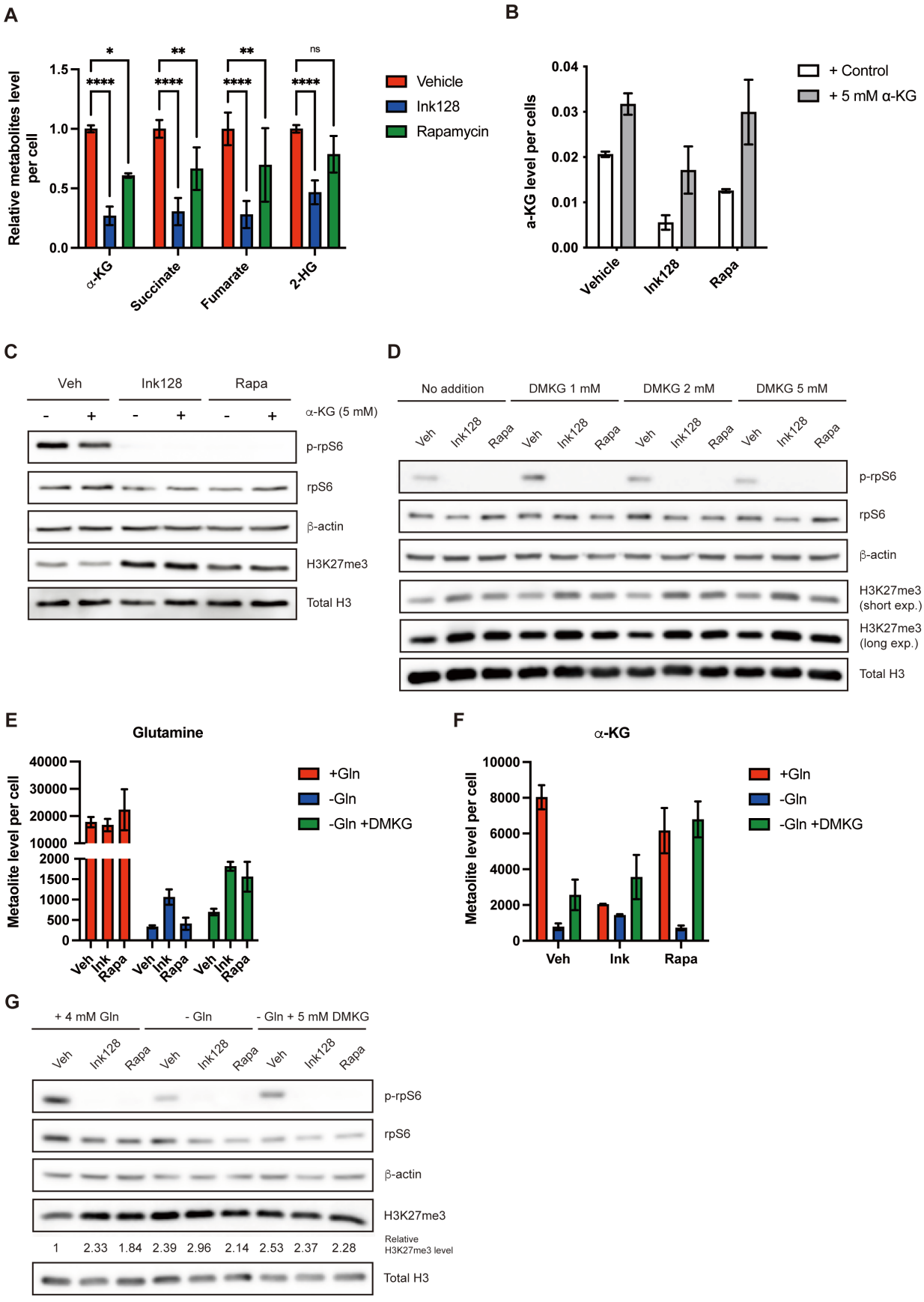
Fig 2.8. The levels and nuclear localization of demethylases are not sufficient to explain the hypermethylation of H3K27 that occurs in response to mTOR inhibition

(A) Expression levels of KDM6A and KDM6B in MCF7 following mTOR inhibitor treatment or serum starvation for 48 h. Stimulation (veh) is used as a control of treatment. Bars represent mean ± SD. (B) Levels of the indicated proteins in MCF7 upon mTOR inhibition were determined by Western blotting. The relative intensity of KDM6A and KDM6B was measured using ImageJ and normalized by the intensity of β-actin, which served as a loading control. (C) Immunoblot analysis of cytoplasmic and nuclear extracts from MCF7 cells treated with mTORi for 48 h. α-tubulin and H3 served as loading controls of cytoplasmic and nuclear proteins, respectively.

4.2. The alterations in α-KG levels are not responsible for the H3K27 hypermethylation caused by mTOR inhibition.

As KDM6A and KDM6B are part of the JmjC domain-containing KDMs, which react with α-ketoglutarate (α-KG) (Franci et al., 2014), we investigated the impact of metabolites on their activity by measuring the levels of metabolites. Since succinate, fumarate and 2HG are structurally related to α-KG, those metabolites are considered as inhibitors of α-KG-dependent dioxygenases, indicating their involvement in demethylation processes (Chowdhury et al., 2011; van der Knaap & Verrijzer, 2016; Xiao et al., 2012). By using GC-MS, we assessed their levels at steady state. As a result, the levels of succinate, fumarate, and 2-HG were reduced after mTOR inhibition (Fig 2.9A). These results suggest that the reduced level of these metabolites did not appear to explain the H3K27 hypermethylation following mTOR inhibition. Furthermore, our findings indicated a decrease in α-KG levels following mTOR inhibition (Fig 2.9A). Our observation led us to formulate a hypothesis that α-KG could be the underlying factor responsible for the elevation in H3K27me3 levels following treatment with mTORi, since α-KG is a known facilitator of demethylation (Chung et al., 2020). To test this hypothesis, we conducted α-KG rescue experiments, where cells were treated with 5 mM α-KG in the presence of mTORi. We confirmed a corresponding increase in intracellular α-KG (Fig 2.9B), but the addition of α-KG did not impact H3K27me3 changes compared to control (no α-KG addition) (Fig 2.9C). To improve bioavailability of α-KG, we conducted a rescue experiment using varying doses of dimethyl α-ketoglutarate (DMKG), a cell-permeable derivative of α-KG. However, our results showed that the addition of DMKG did not have any effect on H3K27me3 levels (Fig 2.9D).

We considered the possibility that the lack of observed effects of α-KG on H3K27me3 could be attributed to the cells already possessing sufficient levels of α-KG before α-KG addition. To investigate this, cells were deprived of glutamine for 4 h and then treated with 5 mM DMKG or untreated for 48 h. Since normal media contains 4 mM glutamine, we also added 4 mM glutamine to confirm the effect of mTOR inhibition under normal conditions. Glutamine deprivation effectively reduced both glutamine and α-KG levels (Fig 2.9E-F). It is worth noting that when cells did not have enough glutamine, the mTOR inhibitors did not reduce α-KG levels as expected (Fig 2.9F). Consistent with previous findings, mTOR inhibition induced H3K27me3, as observed in lanes 1-3 in Fig 2.9G. Furthermore, glutamine deprivation elevated H3K27me3 levels in vehicle-treated cells but not in mTOR-inhibited cells (Fig 2.9G). Despite observing a substantial increase in α-KG upon DMKG addition, DMKG addition had no effect on H3K27me3 levels in either control or mTOR-inhibited cells (Fig 2.9G). Taken together, our α-KG/DMKG addition experiments led us to conclude that α-KG plays a role in the demethylation of H3K27me3. This conclusion is supported by our findings that glutamine deprivation reduces α-KG levels, which subsequently induces H3K27me3, consistent with previous reports (Pan et al., 2016). However, the addition of α-KG does not affect H3K27me3 levels, regardless of mTOR inhibition. Overall, our results suggest that the hypermethylation of H3K27 following mTOR inhibition is independent of the demethylation processes.


A
Relative metabolites level per cell
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α-KG
Succinate
Fumarate
2-HG
Vehicle
Ink128
Rapamycin
B
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0.00
+ Control
+ 5 mM α-KG
Vehicle
Ink128
Rapa
C
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α-KG (5 mM)
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rpS6
β-actin
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D
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DMKG 2 mM
DMKG 5 mM
Veh
Ink128
Rapa
p-rpS6
rpS6
β-actin
H3K27me3 (short exp.)
H3K27me3 (long exp.)
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E
Glutamine
Metaolite level per cell
40000
30000
20000
10000
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1000
500
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+Gln
-Gln
-Gln +DMKG
Veh
Ink
Rapa
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Metaolite level per cell
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+Gln
-Gln
-Gln +DMKG
Veh
Ink
Rapa
G
+ 4 mM Gln
- Gln
- Gln + 5 mM DMKG
Veh
Ink128
Rapa
p-rpS6
rpS6
β-actin
H3K27me3
1 2.33 1.84 2.39 2.96 2.14 2.53 2.37 2.28
Relative H3K27me3 level
Total H3

Fig 2.9. The H3K27 hypermethylation observed upon mTOR inhibition is not mediated by the cofactor of demethylases, α-KG

(A) Quantification of levels at steady state of metabolites from MCF7 cells treated with mTOR inhibitors for 48 h. Metabolites were extracted, profiled by GC-MS, and normalized to cell numbers. Bars represent mean ± SD. ns: no significancy, *P< 0.05, **P<0.01, ****P< 0.0001, one-way ANOVA with Dunnett's post-test compared to vehicle. (B) Quantification of levels at steady state of α-KG from MCF7 cells treated with 5 mM α-KG in the presence of mTORi for 48 h in MCF7. Metabolites were extracted, profiled by GC-MS, and normalized to cell numbers. Bars represent mean ± SD. (C-D) Levels of the indicated proteins in MCF7 treated with 5 mM α-KG (C) or indicated concentration of DMKG (D) in the presence of mTORi for 48 h were determined by Western blotting. β-actin served as a loading control. (E-F) Quantification of levels of glutamine (E) and α-KG (F) at steady state from MCF7 cells treated as indicated. Metabolites were extracted, profiled by GC-MS, and normalized to cell numbers. Bars represent mean ± SD. (G) Levels of the indicated proteins in MCF7 cells treated as described. β-actin served as a loading control. The band intensity was measured by ImageJ and normalized to + 4mM Gln with veh condition.

5. The functional activity of PRC2 is crucial for H3K27 hypermethylation upon mTOR inhibition.

5.1. EZH1 and EZH2 are involved in the mTORi-induced H3K27 hypermethylation but not via alterations in their levels.

We conducted further research to determine whether PRC2, the enzyme responsible for adding methyl groups to H3K27, is responsible for the induction of H3K27 hypermethylation upon mTOR inhibition. We assessed the expression levels of EZH1 and EZH2, enzymes responsible for H3K27 methylation, upon mTOR inhibition. Our findings displayed that serum starvation and Ink128 treatment resulted in a decrease in EZH2 and an increase in EZH1 levels, when compared to control cells (Fig 2.10A). Of note, the effect of rapamycin on both EZH1 and EZH2 was not as pronounced as the effect observed with Ink128 and serum starvation (Fig 2.10A). Furthermore, our results demonstrated corresponding changes in the protein levels of both EZH1 and EZH2 compared to their expression levels (Fig 2.10B). This result is consistent with previous studies that suggest 4E-BP1 to be involved in the translational regulation of EZH2 (Smith et al., 2019). As rapamycin has minimal effects on p-4E-BP1 (Choo et al., 2008), it does not significantly impact EZH2 protein levels (Fig 2.10B). It is reported that EZH1 may be upregulated to compensate for the reduction of EZH2 to maintain the PRC2 activity (Raphael Margueron et al., 2008; Shen et al., 2008). This implies that though mTOR inhibition reduces EZH2, the PRC2 activity can be maintained by increased EZH1. To confirm the significance of EZH1 and EZH2 in H3K27 hypermethylation under mTOR inhibition, we established HCT116 cell lines with knockdowns of either EZH1 or EZH2. Our findings indicated that H3K27me3 levels were reduced following knockdown of both EZH1 and EZH2 (Fig 2.10C-D). Furthermore,

when EZH1 or EZH2 was downregulated via shRNA, the induction of H3K27me3 by Ink128 treatment was abolished (Fig 2.10C-D). This suggests that both EZH1 and EZH2 play roles in H3K27 hypermethylation in response to mTOR inhibition. A similar effect was observed when we treated cells with the EZH2 inhibitor (GSK126) or the EZH1/2 dual inhibitor (UNC1999). Both treatments were able to attenuate the increase in H3K27me3 levels induced by mTOR inhibition (Fig 2.10E). According to these findings, mTOR inhibition alters EZH1/2 that may be required to maintain PRC2 activity under mTOR inhibition.

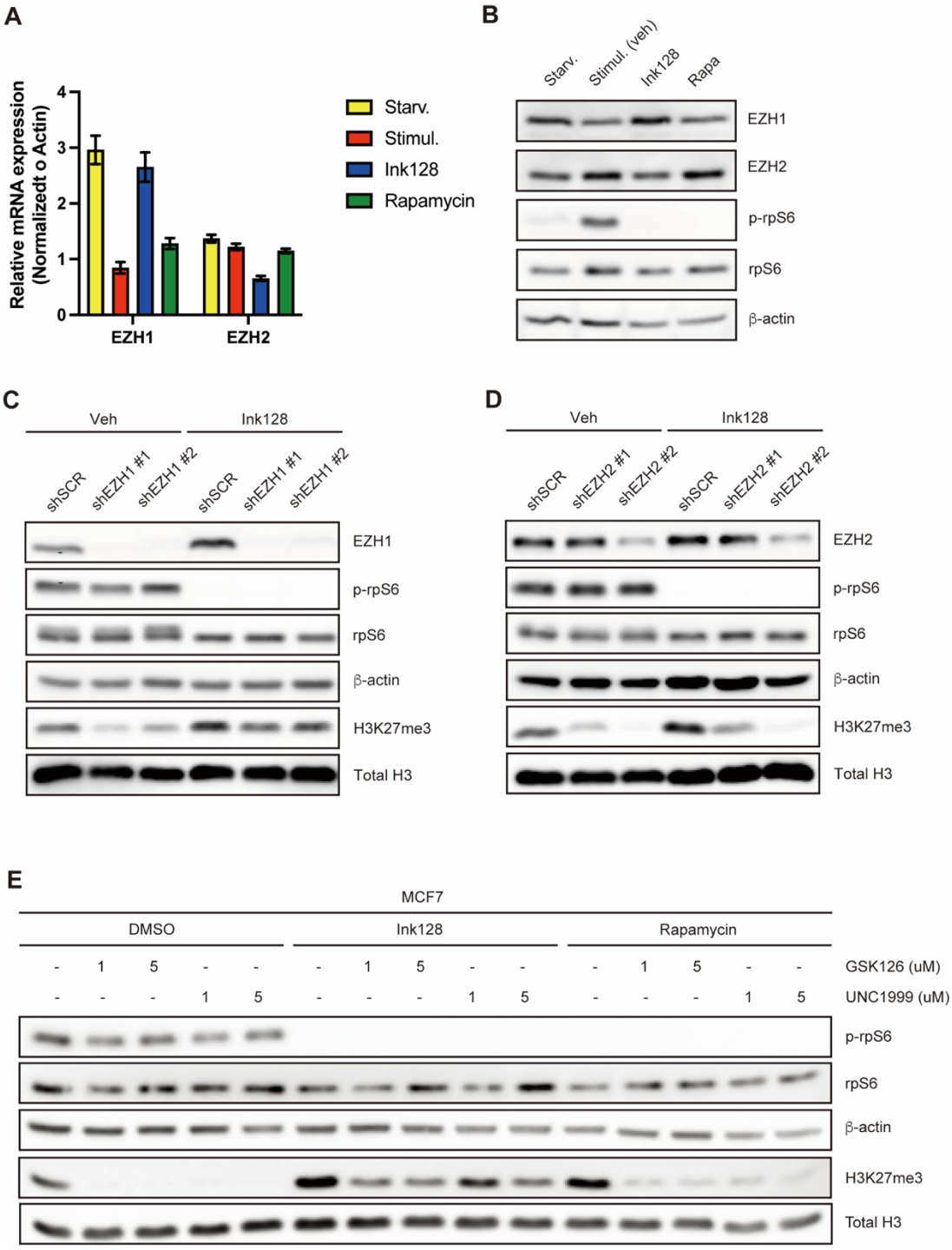

Fig 2.10. EZH1 and EZH2 mediate H3K27me3 induction upon mTOR inhibition, but their levels do not fully explain this effect

(A) Expression levels of EZH1 and EZH2 in MCF7 cells following mTOR inhibitors' treatment or serum starvation for 48 h. Stimulation (veh) was used as a treatment control. (B) Levels of the indicated proteins in MCF7 upon mTOR inhibition were determined by Western blotting. β-actin served as a loading control. (C) Levels of the indicated proteins in HCT116 infected with a scrambled (shSCR) or EZH1-specific shRNA (shEZH1) with vehicle or Ink128 (100 nM) were determined by Western blotting. β-actin served as a loading control. (D) Levels of the indicated proteins in HCT116 infected with a scrambled (shSCR) or EZH2-specific shRNA (shEZH2) with vehicle or Ink128 (100 nM) were determined by Western blotting. β-actin served as a loading control. (E) Levels of the indicated proteins in MCF7 with indicated concentrations of GSK126 or UNC1999 in the presence of mTORi were determined by Western blotting. β-actin served as a loading control.

5.2. EZH2 activity is not regulated by substrate levels, binding to other PRC2 partners, or nuclear localization upon mTOR inhibition.

It is possible that mTOR inhibition affects the activity of PRC2 instead of altering the EZH1/2 protein levels. To further investigate this, we examined the steady state levels of SAM and SAH, which are critical substrates for methyltransferases and essential for their enzyme activity (Dillon et al., 2005). Our findings indicate that mTOR inhibition did not significantly affect the levels of either SAM or SAH (Fig 2.11A-B), nor did it alter the SAM/SAH ratio (Fig 2.11C). This suggests that the activity of H3K27me3 writers is not regulated by the availability of methyl donors in the case of mTOR inhibition.

Multiple research studies have proposed that EZH2 phosphorylation influences the stability, activity, localization, and binding partners of PRC2 components (Li et al., 2020; Yang & Li, 2020). Given that we have established that the protein level of EZH2 cannot account for the induction of H3K27me3 following mTOR inhibition, we proceeded to investigate whether inhibiting mTOR promotes the assembly of PRC2 with other components. Our findings revealed that treatment with Ink128 reduced the binding of core PRC2 components EED and SUZ12, but this change was likely due to a reduction in the corresponding protein levels after Ink128 treatment, rather than directly affecting PRC2 assembly (Fig 2.11D). Though Ink128 treatment reduced binding between EZH2 and RBBP4, another core component of PRC2, without reducing RBBP4 level (Fig 2.11D), this is largely inconsistent with the induction of H3K27me3 upon mTOR inhibition. Therefore, it is unlikely that mTOR inhibition regulates PRC2 assembly to induce H3K27me3.

We also examined the localization of EZH2, as it is crucial for its function as a methyltransferase (Thakar et al., 2021). Our findings indicated that Ink128 treatment resulted in a decrease in the

nuclear fraction of EZH2, while there was no significant change following rapamycin treatment (Fig 2.11E). However, this decrease was also accompanied by a reduction in the total level of EZH2, which is consistent with our earlier observations as shown in Fig 2.10B. These findings suggest that the increase in H3K27me3 levels observed upon mTOR inhibition is unlikely to be caused by changes in either PRC2 assembly or EZH2 localization. Hence, it is necessary to conduct additional research to understand PRC2 activity through alternative mechanisms.

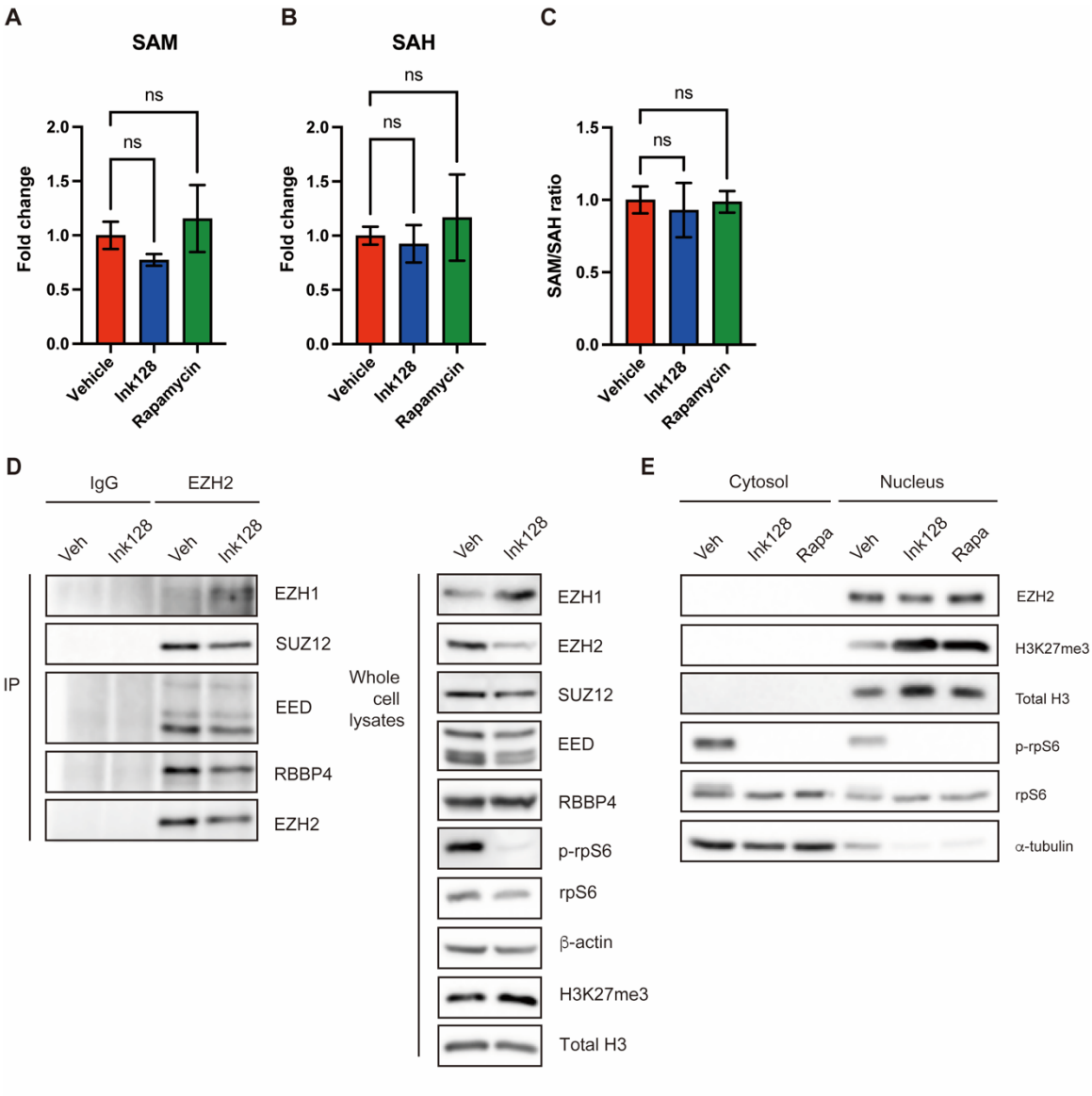

Fig 2.11. mTOR inhibition does not appear to have a direct regulatory effect on EZH2 activity through mechanisms involving SAM, PRC2 assembly, or EZH2 localization

(A-B) Quantification of SAM and SAH levels at steady state from MCF7 cells treated with mTOR inhibitors for 48 h. Metabolites were extracted, profiled by LC-MS, and normalized to cell numbers. ns: no significancy. Bars represent mean ± SD (C) The SAM/SAH ratio was calculated from Fig 2.11A-B. Bars represent mean ± SD (D) Immunoblot analysis of whole cell lysates and anti-EZH2 immunoprecipitates derived from MCF7 cells treated with 100 nM Ink128

for 24 h after overnight serum-starvation. β-actin served as a loading control. (E) Immunoblot analysis of cytoplasmic and nuclear extracts from MCF7 cells treated with mTORi for 48 h. α-tubulin and H3 served as loading controls of cytoplasmic and nuclear proteins, respectively.

5.3. GLP and G9a are not mediators of the induction of H3K27me3 levels resulting from mTOR inhibition.

Given that the H3K9 methyltransferases GLP (also known as euchromatic histone-lysine N-methyltransferase 1; EHMT1) and G9a (also known as euchromatic histone-lysine N-methyltransferase 2; EHMT2) are involved in PRC2-mediated H3K27me2 and H3K27me3 (Meng et al., 2020; Mozzetta et al., 2014), we examined the level of GLP and G9a by Western blotting. Contrary to previous reports averring that mTOR inhibition induces G9a (A. Mushtaq et al., 2023), our findings indicated that the inhibition of mTOR did not lead to a conspicuous change in the level of G9a, despite reducing GLP levels (Fig 2.12A). To determine whether GLP/G9a activity plays a role in H3K27 hypermethylation upon mTOR inhibition, cells were treated with UNC0642, a GLP/G9a inhibitor, in the presence of mTORi or a vehicle (DMSO). We confirmed the efficacy of UN0642 by measuring H3K9me2 levels (Fig 2.12B) as GLP/G9a methylate H3K9me2, not H3K9me3. Our findings show that H3K27me2 and H3K27me3 levels were induced upon mTOR inhibition, whereas inhibition of GLP/G9a activity had no impact on either H3K27me2 or H3K27me3 levels (Fig 2.12B), suggesting that GLP/G9a are not involved in mTOR inhibition-induced H3K27me3 alterations.

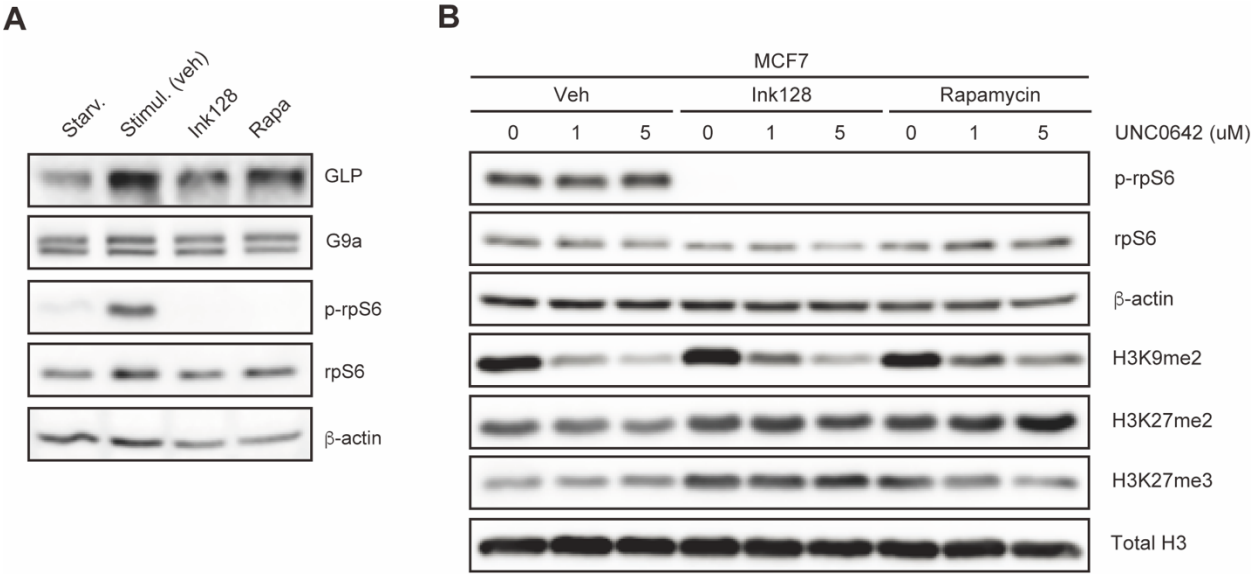

Fig 2.12. The induction of H3K27me3 caused by mTOR inhibition is not mediated by either GLP or G9a

(A) Levels of the indicated proteins in MCF7 upon mTOR inhibition were determined by Western blotting. β-actin served as a loading control. (B) Levels of the indicated proteins in MCF7 treated with indicated concentrations of UNC0642 in the presence of mTORi or a vehicle (DMSO) for 48 h were determined by Western blotting. β-actin served as a loading control.

6. The effect of mTOR inhibition on cell proliferation is partially mediated by induction of H3K27me3.

6.1. Inhibition of mTOR or H3K27me3 modulation reduces cell proliferation.

In order to investigate the consequences of H3K27me3 induction resulting from mTOR inhibition, we first examined the effect of mTOR inhibition on cell proliferation. Consistent with previous reports (Dowling et al., 2010), we found that mTOR inhibitors reduced cell proliferation in MCF7 and HCT116 cell lines, with Ink128 exhibiting a stronger effect than rapamycin (Fig 2.13A-B).

We further investigated the impact of H3K27me3 alteration on cell proliferation. Firstly, we treated MCF7 cells with GSK126 to reduce H3K27me3 levels and observed a dose-dependent decrease in cell proliferation (Fig 2.13C). Secondly, we compared shEZH2 cells to control cells to examine the effect of EZH2 knockdown on cell growth and proliferation, and found that EZH2 knockdown impaired cell proliferation and growth (Fig 2.13D-E). Furthermore, we induced H3K27me3 by treating cells with GSKJ4 and found that cells treated with 5 uM GSKJ4 displayed reduced cell proliferation (Fig 2.13F). Reduced cell proliferation was observed upon both H3K27me3 reduction and induction, emphasizing the significance of comprehending the role of H3K27me3 in a context-dependent manner. Overall, our findings confirm previous body of research demonstrating that blocking mTOR or modulating H3K27me3 levels result in decreased cell proliferation.

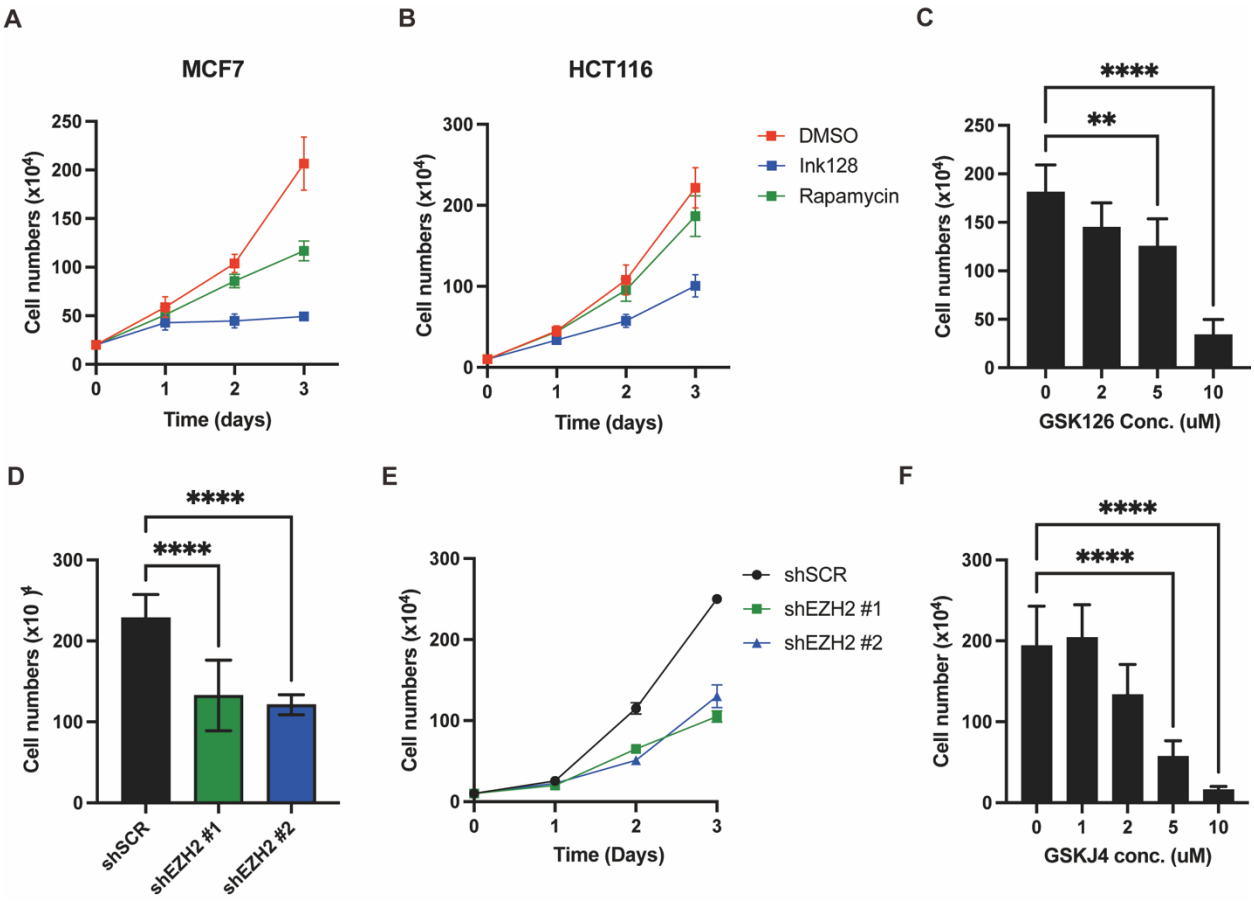

Fig 2.13. Cell proliferation decreases upon mTOR inhibition or H3K27me3 modulation

(A-B) Growth curves of the indicated cancer cell lines treated with vehicle (DMSO), 100 nM Ink128 or 50 nM rapamycin. Bars represent mean ± SD. (C) Cell counting from MCF7 cells treated with indicated concentrations of GSK126 for 72 h. Bars represent mean ± SD. **P<0.01, ****P< 0.0001, one-way ANOVA with Dunnett's post-test. (D) Cell counting from HCT116 cells infected with lentiviruses expressing shScramble (control) or shEZH2 for 72 h. Bars represent mean ± SD. ****P< 0.0001, one-way ANOVA with Dunnett's post-test. (E) Growth curves of HCT116 cells infected with lentiviruses expressing shScramble (control) or shEZH2. Points represent mean ± SD. (F) Cell counting from MCF7 cells treated with indicated concentrations of GSKJ4 for 72 h. Bars represent mean ± SD. ****P< 0.0001, one-way ANOVA with Dunnett's post-test.

6.2. Cells with low H3K27me3 levels are less susceptible to the mTOR inhibitor.

To investigate the effect of H3K27me3 induction on mTOR inhibition-mediated cell proliferation, we utilized a H3K27M model in which H3K27me3 cannot be induced due to a lysine-27-to-methionine substitution. We obtained the DIPG13 cell line which contains the heterozygous H3K27M mutation (Krug et al., 2019) and DIPG13 H3K27M-KO cells, where the K27M mutant allele was removed using the CRISPR/Cas9 system (Ashot S. Harutyunyan et al., 2019). This allowed us to investigate the role of this mutation in a controlled and tumour-relevant setting. First, we confirmed that DIPG13 H3K27M cells are deficient in H3K27me3. This was evidenced by the lack of detectable H3K27me3 signal in control H3K27M cells as compared to H3K27M-KO cells (Fig 2.14A). In line with observations in MCF7 and HCT116 cells, our findings showed that Ink128 treatment induced H3K27me3 in DIPG13 H3K27M-KO cells, while the H3K27me3 signal was still not detectable in H3K27M cells (Fig 2.14A).

To investigate the involvement of H3K27me3 in mTORi-mediated cell proliferation, we next compared the susceptibility of H3K27M and H3K27M-KO cells to Ink128. Importantly, the effects of Ink128 were attenuated in H3K27M cells as compared to H3K27M-KO cells (Fig 2.14B). This suggests that H3K27me3 induction may contribute to the anti-proliferative effects of mTORi.

We further investigated the impact of reducing H3K27me3 levels using an EZH2 inhibitor (GSK126) or EZH2 knockdown with or without Ink128 treatment. Our results revealed that cells treated with GSK126, leading to reduced H3K27me3 levels, exhibited decreased susceptibility to Ink128 (Fig 2.14C). Similarly, shEZH2 cells, which were previously established and have lower H3K27me3 levels, exhibited a reduced response to Ink128 compared to the control cells (shSCR)

(Fig 2.14D). Overall, these observations suggest the anti-proliferative effect of the mTOR inhibitor may, at least in part, dependent on H3K27me3.

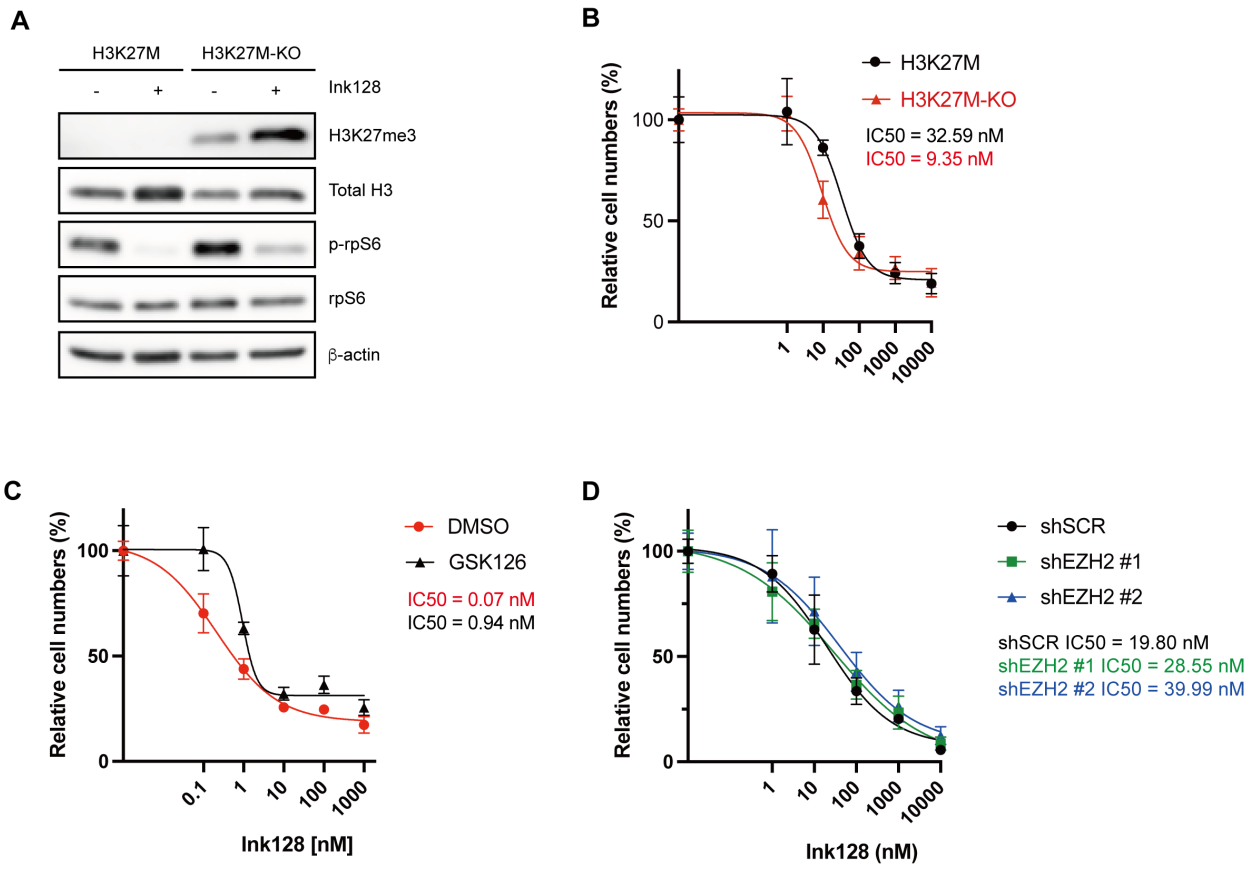

Fig 2.14. The effects of mTOR inhibition on cell proliferation may be in part mediated by H3K27me3 induction

(A) Levels of the indicated proteins in DIPG13 H3K27M and H3K27-KO cells were determined by Western blotting. DIGP13 cells were treated with Ink128 or vehicle (DMSO) for 48 h. β-actin served as a loading control. (B) DIPG13 cells were treated with indicated concentrations of Ink128 for 72 h. Points represent mean ± SD. Cell proliferation was measured using a cell counter, and IC_{50} was calculated by GraphPad. (C) MCF7 cells were treated with indicated concentrations of Ink128 in the presence of DMSO (vehicle) or 2 uM GSK126 for 72 h. Points represent mean ± SD. Cell proliferation was measured using a cell counter, and IC_{50} was calculated by GraphPad. (D) HCT116 cells infected with lentiviruses expressing shScramble (control) or shEZH2 were treated with indicated concentrations of Ink128 for 72 h. Points

represent mean ± SD. Cell proliferation was measured using a cell counter, and IC_{50} was calculated by GraphPad.

CHAPTER 4. Discussion and Conclusion

1. Summary

We investigated the role of mTOR on histone methylation. Using TSC2-null MEFs as a model of constitutive mTORC1 activation, we observed selective induction of H3K27me3, a histone methylation mark associated with gene repression. Our study confirmed that constitutive mTORC1 activation elevated H3K27me3 through the 4E-BP1/EZH2 axis, consistent with previous reports (Smith et al., 2019), and demonstrated the essential role of EZH2 in the hypermethylation of H3K27 resulting from constitutively active mTORC1. Surprisingly, independent of TSC2, mTOR inhibition led to an increase in H3K27me3 levels. Although we observed an elevation of H3K9me3 levels in MCF7 and HCT116 cells upon mTOR inhibition by Western blotting, we failed to reproduce these effects using IF or ChIP-seq. Thus, we turned our attention towards the induction of H3K27me3 following mTOR inhibition. Our results suggest that the inhibition of both mTORC1 and mTORC2 may be implicated in inducing H3K27me3. Notably, changes in H3K27me3 levels caused by mTOR inhibition did not appear to be secondary to the effects of mTOR on cell cycle.

To understand how mTOR inhibition leads to H3K27me3 induction, we firstly investigated the expression and protein levels of demethylases, KDM6A and KDM6B. Our findings revealed that the observed H3K27 hypermethylation after mTOR inhibition could not be attributed to alterations in KDM6A/6B levels or changes in their nuclear localization. We also examined the impact of metabolites on KDM6A/6B demethylase activity. Though the α-KG level was also reduced upon mTOR inhibition, the addition of α-KG or DMKG did not affect H3K27me3 changes compared to control (nor α-KG or DMKG addition). These findings suggest that the

induction of H3K27 hypermethylation upon mTOR inhibition appears to be independent of demethylation processes.

To investigate the potential involvement of PRC2 in the H3K27me3 induction following mTOR inhibition, we examined the role of EZH1 and EZH2. Our findings revealed that both EZH1 and EZH2 may play a role in this process, with EZH2 being more potent than EZH1. Furthermore, our investigations showed that the decrease in EZH2 protein levels following mTOR inhibition did not account for the observed elevation in H3K27me3 levels. This implies that mTOR inhibition may regulate the activity of PRC2 rather than affect the protein levels of EZH1/2. Therefore, we examined the regulation of EZH2 activity through substrate levels, binding to other PRC2 partners, or nuclear localization, in the context of mTOR inhibition. Our results showed that mTOR inhibition did not affect the levels of SAM and SAH, which are critical substrates for methyltransferases. Additionally, inhibiting mTOR did not promote the assembly of EZH2 with other PRC2 components, and the decrease in nuclear fraction of EZH2 following mTOR inhibition was unlikely to be the cause of the observed increase in H3K27me3 levels. Other potential regulators of PRC2-mediated H3K27me3, viz. GLP/G9a, were examined, but did not appear to be responsible for the hypermethylation of H3K27me3 induced by mTOR inhibition. Overall, our study highlights the need for further research in order to fully understand the mechanism behind the increase in H3K27me3 levels observed upon mTOR inhibition.

Lastly, we investigated the impact of mTOR inhibition and H3K27me3 on cell proliferation. Herein, using H3K27M mutant and EZH2 inhibited cells, we found that that cells with impeded H3K27me3 exhibited a decreased vulnerability to mTOR inhibition, indicating that H3K27me3 induction may partially contribute to mTORi-induced cell proliferation. Collectively, mTOR

dysregulation induces H3K27me3, and when mTOR is inhibited, H3K27me3 induction contributes, to some extent, to mTORi-mediated cell proliferation (Fig 3.1).

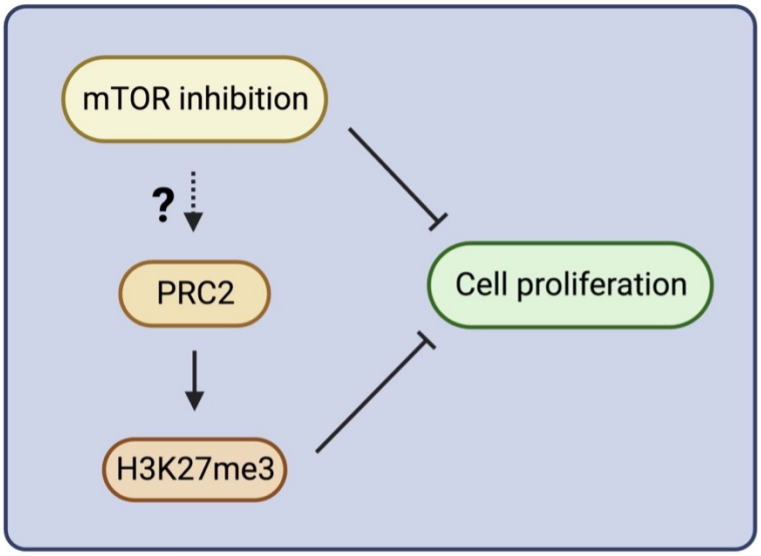

Fig 3.1. Schematic diagram illustrating mTORi-dependent regulation of cell proliferation via H3K27me3 induction

2. Further investigation is needed to elucidate the specific mechanism by which mTOR inhibition regulates PRC2.

Our findings indicated that the induction of H3K27me3 by mTOR inhibitors is mediated through PRC2, as demonstrated by the reversal of this effect upon EZH2 inhibition or knockdown. Our hypothesis is supported by the observation that H3K27M mutated cells did not respond to Ink128 treatment by inducing H3K27me3, while H3K27M-KO cells did, further reinforcing our idea. Whereas the precise mechanism by which the H3K27M mutation reduces H3K27me3 remains unclear, PRC2 is known to be involved in this regulation (Margueron & Reinberg, 2011). However, we still could not find how mTOR inhibition regulates PRC2 activity.

As previously discussed, PRC2 is regulated in a number of ways, notably through its assembly and localization (Yang & Li, 2020). Therefore, it is possible that other mechanisms are involved in the hypermethylation of H3K27 following mTOR inhibition. For example, it is possible that the inhibition of mTOR leads to the regulation of other histone modifications that promote the H3K27me3 levels. PRC1-mediated H2AK119ub1 is known to contribute to H3K27me3-mediated gene silencing, as evidenced by the reliance of PRC2 binding and H3K27me3 deposition on PRC1 catalytic activity (Dobrinić et al., 2021). Moreover, H2BS36 phosphorylation mediated by nuclear S6K1 facilitates the recruitment of EZH2 to H3, leading to H3K27 methylation (Yi et al., 2016). However, in our study, treatment with mTOR inhibitors resulted in the reduction of p-rpS6, a downstream target of S6K. It is possible that mTOR inhibition may be involved in the translocation of S6K1, but this remains contextually uncertain. Since the involvement of nuclear mTOR has been established (Audet-Walsh et al., 2017; Chaveroux et al., 2013; Torres & Holz, 2021), it is plausible that nuclear mTOR interacts with

H3K27me3 modifiers or histones themselves to trigger H3K27me3 induction upon mTOR inhibition.

Inhibition of mTOR can lead to the induction of H3K27me3 by regulating other transcriptional factors like c-MYC. In cells with $BRAF^{V600E}$ mutation, which is the most common genetic alteration in the MAPK/Erk pathway, hyperactivation of MAPK/Erk signaling occurs, leading to tumorigenesis and increased H3K27me3 levels (Qu et al., 2017). In this model, c-MYC, a downstream key effector of $BRAF^{V600E}$, plays a crucial role in H3K27 hypermethylation by regulating PRC2 components at both transcriptional and post-transcriptional levels, by repressing miR-26a, miR-200b, and miR-155 (Qu et al., 2017). However, previous reports indicate that inhibition of mTORC1 leads to a decrease in c-MYC mRNA translation (Csibi et al., 2014). Furthermore, our findings suggest that c-MYC may not play a role in the mTOR-mediated regulation of H3K27me3, as evidenced by the significant decrease in PRC2 components, such as EZH2 and SUZ12, with asTORi (Ink128) treatment (Fig 2.11D).

PRC2 can also be regulated by HOTAIR, a long non-coding RNA transcribed from the HOXC gene cluster on chromosome 12 (Rinn et al., 2007). HOTAIR is known to recruit PRC2 to specific genomic sites, leading to the addition of H3K27me3 (Tsai et al., 2010). While some studies have shown that HOTAIR activates the PI3K/AKT/mTOR pathway in breast cancer (Li et al., 2019; Sadeghalvad et al., 2022), it is not clear whether mTOR plays a role in regulating HOTAIR.

Our study primarily focused on the activity of EZH2. The effects of mTOR inhibition may be however mediated by other components of PRC2. PRC2 consists of core components such as SUZ12, EED, and RBBP4, as well as accessory components like JARID2, AEBP2, EPOP, and PCLs, all of which are supposed to be implicated in regulating PRC2 outputs (Yang & Li, 2020).

While we observed a reduction in the level of SUZ12 and EED following Ink128 treatment in Fig 2.11D, we did not investigate their activity or that of other components such as JARID2, AEBP2, EPOP and PCLs. Therefore, it may be warranted to study the regulation of PRC2 components to determine the mechanism by which mTOR inhibition induces H3K27 hypermethylation.

3. Discrepancies in results reported by other research groups

Consistent with the findings of the Brown group (Spangle et al., 2016), we observed no changes in H3K4me3 levels upon mTOR inhibition. However, in contrast to other studies (Harachi et al., 2020; Ni et al., 2022), we observed a induction in H3K27me3 levels upon mTOR inhibition. The Shibata group reported that mTORC1 upregulates the protein level of EZH2 in human glioblastoma, while mTORC2 regulates the production of SAM (Harachi et al., 2020). This cooperative regulation results in H3K27 hypermethylation, which promotes tumour cell survival both *in vitro* and *in vivo* xenografted mouse tumour models (Harachi et al., 2020). It has also been reported that loss of Raptor reduces H3K27me3 in β-cells by decreasing EED, a core subunit of PRC2 (Ni et al., 2022). Our findings differ from these reports. While we also observed a reduction in EZH2 when asTORi (Ink128) was employed, we found that mTOR inhibition led to an increase in H3K27me3 levels, independent of EZH2 protein levels.

We propose that the different effects of mTOR inhibition on H3K27me3 may be due to the context under which these phenomena are studied. Our research examined the induction of H3K27me3 following mTOR inhibition in MEFs, breast cancer, colorectal cancer, and DIPG, but we have not yet examined it in pancreatic β-cells or glioblastoma. Therefore, it is necessary to test different models to generalize the effect of mTOR inhibition on H3K27me3.

Furthermore, we believe that the discrepancies between our results and those presented in previous reports may be attributed to differences in the duration of mTOR inhibition. Specifically, we utilized inducible knockout systems that require more time to deplete Raptor or Rictor, whereas previous studies used siRNA to target these proteins (Harachi et al., 2020). Additionally, we observed changes in histone methylation after at least 24 h of treatment following overnight serum starvation, which implies that mTOR had been inhibited for approximately 40 h. Given the complex interplay between mTOR signaling and other pathways, it is possible that different results may be obtained when altering H3K27me3 levels. It is also worth noting that we used Ink128 and serum starvation as alternative methods to inhibit mTOR, which are more potent in inhibiting phosphorylation of 4E-BP1 and mTORC2, whereas previous studies employed rapamycin as an mTOR inhibitor.

4. The intricate nature of mTOR signaling and its interplay with other signaling pathways

As mentioned earlier, the mTOR signaling pathway is intricately interconnected with other signaling molecules such as AMPK and AKT. While we did not assess AMPK activity in our model, there is evidence suggesting that mTOR inhibitors increase AMPK activity (Dagon et al., 2012; Ling et al., 2020). Based on previous research indicating that mTOR inhibitors may affect AMPK activity, we considered the possibility that AMPK could play a role in the induction of H3K27me3 following mTOR inhibition. The Wei group found that AMPK-null cells exhibit elevated levels of H3K27me3 compared to WT cells, which is attributable to AMPK-mediated phosphorylation of EZH2 at the T311 site (Wan et al., 2018). Phosphorylation of EZH2 at T311 disrupts the binding between EZH2 and SUZ12, resulting in a reduction of H3K27me3 levels (Wan et al., 2018). Considering that the activation of AMPK is known to inhibit mTOR signaling

(Gwinn et al., 2008), our observation of increased H3K27me3 levels in mTOR-inhibited cells contrasts with the expected outcome in AMPK-null cells. Furthermore, we found that mTOR inhibition did not alter the binding of EZH2 with SUZ12, indicating that AMPK-mediated phosphorylation of EZH2 at the T311 site is unlikely to be involved in mTORi-induced H3K27me3. In our study, we employed rapamycin and Ink128 to inhibit mTOR. As illustrated in Fig 2.3B-C, long-term rapamycin treatment increases AKT S473 phosphorylation, while Ink128 reduces it. Nevertheless, we found that both treatments induce H3K27me3, thereby suggesting that AKT is not likely to play a role in the induction of H3K27me3 following mTOR inhibition.

5. An interdependent relationship between H3K27me3 modifiers and mTOR signaling

Recent studies have suggested that there is a reciprocal relationship between H3K27me3 and mTOR signaling. As we observed, mTOR can modulate H3K27me3-associated enzymes like EZH2, thereby influencing H3K27me3 levels. Conversely, the modifiers of H3K27me3 have the potential to regulate mTOR signaling by impacting the expression of key signaling molecules including TSC2 (Wei et al., 2015), PTEN (Jarome et al., 2018), IGF1R and MYC (Kosalai et al., 2019). For instance, EZH2 activates the mTOR pathway via repressing TSC2 (Wei et al., 2015). In male rats, retrieval of contextual fear memory leads to an increase in H3K27me3 levels through upregulation of Ezh2, resulting in transcriptional silencing of the *Pten* gene, a potent inhibitor of AKT-mTOR-dependent signaling in the hippocampus (Jarome et al., 2018). The authors revealed that knockdown of Ezh2 attenuates the increases in AKT and mTOR phosphorylation following retrieval, which can be restored by simultaneous reduction of Pten, suggesting that H3K27me3 regulates AKT-mTOR phosphorylation by repressing PTEN (Jarome et al., 2018). Furthermore, EZH2 directly binds to the *IGF1R* promoter along with MYC and

upregulates *IGF1R* expression, leading to downstream PI3K-mTOR activation (Kosalai et al., 2019). In addition, KDM6A directly binds to DEPTOR, a negative regulator of mTORC1 and mTORC2, leading to diminished activity of both complexes resulting in attenuated tumour progression in liver cancer (Revia et al., 2022). Altogether, the interaction between H3K27me3 modifiers and mTOR signaling is complex and relies on the specific circumstances, underscoring the importance of considering this interplay when evaluating the effect of mTOR dysregulation on H3K27me3.

6. Potential mechanisms by which H3K27me3 mediates the anti-proliferative effects of mTOR inhibitors

mTOR regulates cell proliferation via several mechanisms such as regulating cell cycle progression, apoptosis, and autophagy (Zou et al., 2020). mTOR inhibition induces cell cycle arrest at the G1/S phase (Decker et al., 2003; Dowling et al., 2010; Hleb et al., 2004; Jiang et al., 2003; Takuwa et al., 1999). Furthermore, mTOR has dual roles in apoptosis, serving both as an inhibitor and an inducer of programmed cell death in a context-dependent manner (Chatterjee et al., 2016). While rapamycin derivative CCI-779 induced cell proliferation and reduced apoptosis in prostate cancer (Neshat et al., 2001), rapalogs (everolimus and temsirolimus) trigger the activation of the death receptor pathway and cause apoptosis in colon cancer cells (He et al., 2016). In addition, mTOR is a pivotal regulator of autophagy. It inhibits autophagy via several factors such as TFEB (Napolitano et al., 2018), AMBRA1, and ULK1 (Kim et al., 2011; Nazio et al., 2013). However, the relationship between autophagy and cell death is intricate and not yet fully comprehended (Denton & Kumar, 2019). Although autophagy can induce cell death in some cases (Dasari et al., 2017; Elgendy et al., 2011; Liu et al., 2013), it can also function as a

protective mechanism, safeguarding cells from stress and harm (Degenhardt et al., 2006; Deretic, 2011).

It is noteworthy that PRC2 is involved in many mTOR-regulated biological functions (Parreno et al., 2022). EZH2 is downstream of the pRB-E2F pathway, which plays a critical role in regulating progression through the mammalian cell cycle (A. P. Bracken et al., 2003). In addition, suppressing EZH2 in cholangiocarcinoma cells leads to elevated apoptosis and a halt in the G1 phase of the cell cycle by upregulating p16 and p21. This, in turn, facilitates the progression of cholangiocarcinoma cells (Nakagawa et al., 2013). Furthermore, it has been reported that EZH2 inhibits autophagy via activating mTORC1 signaling in a TSC2-dependent manner (Wei et al., 2015). Given that both mTOR inhibitors and EZH2 inhibitors induce cell cycle arrest (Dowling et al., 2010; T. P. Liu et al., 2015), it is unlikely to observe a beneficial effect on cell proliferation from EZH2 inhibition following mTOR inhibition. Both mTOR inhibition and EZH2 inhibition have been shown to induce autophagy (T. P. Liu et al., 2015; Zullo et al., 2014), and it is plausible that the induction of autophagy by mTOR inhibitors and EZH2 inhibitors could promote cell proliferation considering that autophagy provides fuel and support for the growth of cancer cells (Yun & Lee, 2018).

Differentiating the role of mTOR in H3K27me3 modulation from its involvement in translation and metabolic regulation is essential (Papadopoli et al., 2019). To achieve this, it would be valuable to identify the specific targets of mTOR inhibitor-induced H3K27me3 and manipulate those targets using gene-editing tools. Although the correlation between RNA-seq and ChIP-seq was very weak, we were able to identify a few genes whose RNA levels correlated with H3K27me3 changes (referred to as target genes). To validate these findings, we will begin with ChIP-qPCR and RT-qPCR. Additionally, we plan to treat cells with an EZH2 inhibitor or a

demethylase inhibitor to observe how gene expression responds to H3K27me3 modulation. To distinguish changes resulting from mTOR's involvement in translation and metabolic regulation, we will select certain genes whose RNA levels were not correlated with H3K27me3 alterations but are known to be regulated by mTOR. We will then conduct the same validation experiments as we will for the target genes. Lastly, after validating the target genes, we will manipulate their levels to investigate whether these genes are responsible for mediating the anti-proliferative effects of mTOR inhibitors.

7. Insight into pre-clinical strategies of mTOR and EZH2 inhibition in cancer

mTOR is considered to be an attractive target for cancer treatment, especially for cancers with high mTOR signaling caused by genetic or metabolic abnormalities (Hua et al., 2019). In both *in vitro* and *in vivo* studies, rapamycin effectively inhibits mTORC1 activity, leading to reduced growth of cancer cells (Imrali et al., 2016; Semlali et al., 2022). Consequently, several inhibitors targeting mTOR have been developed and utilized as cancer therapies, with ongoing clinical trials exploring their efficacy (Hua et al., 2019). Despite initially promising results, mTOR inhibitors have not yet achieved their full therapeutic potential due to several reasons. Firstly, inhibiting mTOR triggers various feedback loops that activate upstream signaling pathways, ultimately enhancing the survival and metastasis of cancer cells (Tian et al., 2019). Additionally, mTORC1 suppresses autophagy, and using an mTOR inhibitor may induce autophagy, thereby promoting cancer cell survival, as observed with AZD8055 (Tao et al., 2018). Inhibition of mTOR also results in promoting plasticity and maintains stem-cell-like characteristics in breast cancer cells. This unexpected consequence can lead to the spread of a more aggressive form of

the disease (Jewer et al., 2020). In light of these factors, having a thorough understanding of the specific context is essential before contemplating the use of an mTOR inhibitor.

H3K27me3 dysregulation is common in cancer. EZH2, a crucial component of PRC2, acts as an oncogene, and its abnormal overexpression and dysfunction have been observed in a range of solid tumours, including breast, ovarian, pancreatic, prostate, bladder, and renal cancers (Adrian P. Bracken et al., 2003; Curtis et al., 2012; Hinz et al., 2008; Lee & Choe, 2012; Rao et al., 2010; Saramäki et al., 2006). Additionally, demethylases such as KDM6A and KDM6B, which play a role in regulating H3K27me3, are frequently dysregulated in several cancer types (Dalgliesh et al., 2010; Robinson et al., 2012; Ryuma Tokunaga et al., 2016; van Haaften et al., 2009), although their biochemical regulation remains uncertain. The relationship between cancer and H3K27me3 levels is complicated and can vary depending on the specific type of cancer, given the complex regulation of H3K27me3 through both methyltransferases and demethylases (Das & Taube, 2020).

Specifically, H3K27M mutations are present in almost 80% of DIPGs (Gessi et al., 2015), which results in global hypomethylation of H3K27 by inhibiting PRC2 (Mohammad et al., 2017). Since approximately 50% of DIPGs have been found to harbour amplifications in the signaling pathway involving RTK/PI3K/AKT/mTOR, the latter's downstream effector pathway PI3K/AKT/mTOR has been targeted with single agents (Paugh et al., 2011). Although rapamycin and everolimus were found to be ineffective against DIPG cells in a screening study (Grasso et al., 2015), two clinical trials (NCT02133183 and NCT02142803) are currently underway to investigate the efficacy of Ink128, in treating adult glioblastoma by targeting and suppressing mTORC1/2 while also being able to penetrate the brain. As of yet, there have been no clinical studies conducted with Ink128 specifically for pediatric brain tumours. Not only mTOR

inhibition, but EZH2 inhibition has also gained significant attention as a potential modality to treat DIPGs. Cells with H3K27M mutation show a decrease in H3K27me3 levels throughout the genome by blocking the activity of PRC2 (Mohammad et al., 2017). However, some genes still maintain H3K27me3 marks due to a remaining PRC2 activity. This remaining activity is necessary for the growth of DIPG tumours expressing H3K27M (Mohammad et al., 2017). Therefore, inhibiting EZH2 could be a promising therapeutic approach for treating these tumours. Our research suggests that the effectiveness of mTOR inhibitors in reducing cell growth may be less significant in DIPG patients who harbour the H3K27M mutation, in comparison to those who lack the mutation. This finding should be considered when evaluating treatment alternatives for these patients, particularly given the attention towards using mTOR inhibitors and EZH2 inhibitors to treat gliomas.

Pursuant to our proposal that mTORi-induced H3K27me3 plays a role in mediating the anti-proliferative effect of mTOR inhibition, it is possible that cancers with high levels of H3K27me3 would be more responsive to mTOR inhibitors. Notably, EZH2 gain-of-function (GOF) mutations have been identified in several cancers, including lymphoma and melanoma (Hodis et al., 2012; Souroullas et al., 2016; Yap et al., 2011). Previous studies have shown that all reported GOF mutation sites on EZH2 are located within the SET domain, which leads to an increase in global H3K27me3 levels (M. T. McCabe et al., 2012; Morin et al., 2010). Several GOF mutations on EZH2, such as Y111N, F120L, and Y661D, have been reported to cause drug resistance to SAM-competitive EZH2 inhibitors without affecting the enzymatic activity (Baker et al., 2015; Gibaja et al., 2016). To address this issue, EED inhibitors have been developed to block PRC2 allosterically and effectively inhibit PRC2 with EZH2 inhibitor-resistant mutations

(Ma et al., 2022; W. Qi et al., 2017). Our results propose that mTOR inhibitors may be more effective against cancers with EZH2 GOF mutations compared to those without such mutations.

8. Limitations and future studies

The research we conducted was limited to using TSC2-null MEFs as a representation of constitutively activated mTORC1. Additionally, these MEFs lack p53, which prevented the early senescence of TSC2 KO MEFs (H. Zhang et al., 2003). As a result, we were unable to determine the significance of p53 in this particular model. Therefore, it is necessary to confirm our findings in other models with high mTORC1 activity such as overexpressing RagA and RagC, which are GTPase that activate mTORC1 in response to amino acids (Kim et al., 2008; Sancak et al., 2008). Moreover, it is crucial to account for both mTORC1 and mTORC2 activation when examining the effect of mTOR on histone methylation. However, the mechanism to selectively activate mTORC2 remains unclear, necessitating further investigation into its regulation.

To improve the validity of our findings, it would be helpful to examine the effect of mTOR on H3K27me3 within the same cellular context. One approach could involve using comparable non-transformed and transformed cells, such as MCF-10A and MCF7 cells (Spink et al., 2006) or NMuMG and NMuMG–NT2197 cells (Ursini-Siegel et al., 2008), instead of relying on two different models (TSC2-null MEFs for mTORC1 activation and cancer cells for mTOR inhibition) for comparison.

We measured the levels of histone methylation using Western blotting, ChIP-seq, IF, and flow cytometry. As observed with H3K9me3, the results can vary depending on the detection methods. Therefore, it is crucial to employ multiple methods to assess alterations in histone methylation. Furthermore, ChIP assays often yield low signals compared to controls, resulting in

inconclusive data. Consequently, alternative methods for detecting histone methylation, such as mass spectrometry and CUT&RUN, should be considered.

There are two naturally occurring forms of 2HG: R-2HG and S-2HG. However, standard analytical techniques for detecting 2HG are unable to distinguish between the signals of R-2HG and S-2HG because they are identical in their physical and chemical properties (Cheng et al., 2015). As a result, the sum of the two metabolites is typically measured (Gross et al., 2010; Wang et al., 2013). The IDH mutant enzyme is solely responsible for producing R-2HG (Dang et al., 2009), which is regarded as an oncometabolite that promotes tumourigenesis by inhibiting α-KG-dependent dioxygenases through epigenetic alterations (Chowdhury et al., 2011; Xu et al., 2011). Due to this constraint of the analytical methods, we were unable to determine whether the measured 2HG level was R-2HG or S-2HG.

As epigenetic changes occur in the nucleus (Berger et al., 2009), it is important to consider not only the total levels of SAM and SAH but also their relative abundances. Since we used cell extracts, the metabolite levels reflect their total content, irrespective of subcellular localization. To gain a better understanding of the role of SAM and SAH, it would be beneficial to fractionate nuclear and cytoplasmic extracts and analyze them separately to measure SAM and SAH levels. This approach will help us determine whether alterations in the methyl donor pool play a role in the change in H3K27me3 levels.

While we used various cancer types to observe the effects of mTOR inhibition on histone methylation, we did not test all cancer types, making it difficult to generalize the impact of mTOR inhibition in cancer. Moreover, the majority of experiments were conducted in cell line models, highlighting the need for better preclinical models such as mouse models to develop this research in a therapeutic setting. To confirm our results, we can employ xenograft models using

H3K27M and H3K27M-KO DIPG13 cells. After implanting the tumor, mice will be treated with Ink128, which can penetrate the blood–brain barrier in supratherapeutic concentrations. We will then compare their response to Ink128 by measuring tumor size and volume. Conversely, we can utilize mice with WT EZH2 and mice with EZH2 GOF mutations, characterized by high H3K27me3 levels, and treat them with an mTOR inhibitor to observe whether they exhibit increased sensitivity to the mTOR inhibitor.

As depicted in Fig 2.3A and previously reported by other researchers (Spangle et al., 2016; Wan et al., 2018), changes in histone methylation occurred at a slower pace when compared to effectors in signal transduction such as p-rpS6 and p-4E-BP1. Despite the ability of Ink128 to rapidly inhibit mTOR activity within 4 h (Fig 2.3A), it is possible that the modulation of H3K27me3 following mTOR inhibition may involve indirect regulatory processes. To determine the kinetics of H3K27me3 alteration following mTOR inhibition, it will be necessary to monitor its level at multiple time points. Additionally, employing drugs that target AKT, AMPK, and MAPK/ERK signaling pathways will provide a more comprehensive understanding of their involvement in mTOR inhibition-induced H3K27 hypermethylation.

While we found that EZH2 knockdown can reverse the effect of mTOR inhibition on H3K27me3, suggesting the involvement of PRC2, the mechanism by which PRC2 activity is regulated upon mTOR inhibition remains unclear. As previously mentioned, the activity of PRC2 can be modulated by various factors, including its components, non-coding RNAs such as HOTAIR, post-translational modifications, and other histone modifications. In light of the various factors that can regulate PRC2 activity, it is essential to conduct an initial screening of related gene and protein levels. This can be achieved by utilizing publicly available data sets such as RNA-seq, proteomics, and phosphoproteomics data.

In our investigation of the impact of H3K27me3 in the context of mTOR dysregulation, we did not explore the role of mTORC1 hyperactivation further. This was because TSC2 KO cells did not demonstrate alterations in cell growth under normal serum conditions (10% FBS) compared to TSC2 WT cells, despite their high mTORC1 activity (Hongbing Zhang et al., 2003). As a result, we opted not to explore the impact of mTORC1 hyperactivation on cell proliferation, as it did not produce any alterations in this model, unlike mTOR inhibition.

Given that mTOR inhibitors can influence cell proliferation through various mechanisms as discussed earlier and that the regulation of EZH2 is gene-specific and context-dependent (Duan et al., 2020), it is essential to conduct a further analysis of ChIP-seq and RNA-seq data to differentiate the effect of mTOR on epigenetic regulation from other factors. This analysis will provide insights into how mTOR inhibitors and EZH2 inhibitors influence cell proliferation by assessing their impact on specific target genes.

9. Concluding remarks

The mTOR signaling pathway is involved in multiple biological processes, including cell proliferation, survival, metabolism, autophagy, and stemness (Papadopoli et al., 2019). Recently, it has been discovered that mTOR regulates epigenetic modifiers or the production of SAM or α-KG, which affects the epigenetic landscape (Morita et al., 2013; Smith et al., 2019; Wan et al., 2017; Zeng et al., 2019). In our study, we investigated the contribution of mTOR to histone methylation, and observed that mTORC1 hyperactivation induces H3K27me3 through the 4E-BP1/EZH2 axis, while mTOR inhibition also leads to increased H3K27me3 levels. We determined that the induction of H3K27me3 is not driven by cell cycle progression, demethylation processes, or alterations in EZH1/2 protein levels, but rather by the activity of

EZH2, underscoring the importance of PRC2 in this mechanism. Additionally, our results demonstrated that the induced H3K27me3 may contribute a role in the inhibition of cell proliferation mediated by mTOR inhibition. These findings establish a basis for future investigations focused on elucidating the involvement of mTOR in epigenetic alterations in cancer.

CHAPTER 5. Bibliography

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