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Role of PRMT5 in ULK1-Mediated Autophagy and Breast Cancer Therapy

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies

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Finally, I dedicate this achievement to my deceased mom, Ama Achiaa, who died when I was young – she has been my inspiration.

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List of Abbreviations

Hsp70	Heat-shock 70 kDa protein
G3BP1	Ras-GAP SH3-binding protein 1
WDR77	WD repeat domain 77
GAR or RGG/RG	Glycine and arginine-rich regions
PHF1	Plant homeodomain (PHD) finger protein 1
CUL4B	Cullin 4B
FXBW7	F-box and WD repeat domain containing 7
MEN1	Mul1ple endocrine neoplasia type 1
ST7	Suppression of tumorigenicity 7
NM23	Nonmetastatic 23
RBL2	RB transcriptional corepressor like 1
RBL1	RB transcriptional corepressor like 1
RB1	Retinoblastoma protein 1
CDT1	Chromatin licensing and DNA replication factor 1
DNMT3A	DNA methyltransferase 3A
FGFR3	Fibroblast growth factor receptor 3
eIF4E	Eukaryotic translation initiation factor 4E
Sp1	Specificity protein 1
Brg1	Brahma-related gene-1
JDP2	Jun dimerization protein 2
RNF168	Ring finger protein 168
TGFβ-	Transforming growth factor-β
EMT	Epithelial mesenchymal transition
SNAIL	Snail family transcriptional repressor 1
GADD45	Growth arrest and DNA damage inducible alpha
APAF-1	Apoptotic protease activating factor 1
p73	Tumor protein 73
KLF4	Krüppel-like factor 4
VHL/VBC	Von hippel-lindau tumor suppressor/ pVHL-elongin B-C
GSK3β	Glycogen synthase kinase 3 beta-
ETS	Erythroblast transformation specific
ERG	ETS-related gene
DLBCL	Diffuse large B cell lymphoma
SMN	Survival motor neuron
mdm4	mouse double minute 4
SRSF1	Serine and arginine rich splicing factor 1
ZNF326	zinc finger protein 326
SENP7	Sentrin-specific protease 7
MECOM	MDS1 and EVI1 complex locus
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
PTEN	Phosphatase and Tensin Homolog deleted

BCL2	B-cell lymphoma 2
ITCH	Itchy E3 ubiquitin protein ligase
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
DISC	Death induced signaling complex formation
mTOR	Mammalian target of rapamycin
RPS10	Ribosomal protein S10
IRES	Internal ribosome entry site
AXIN2	Axis inhibition protein 2
WIF1	WNT inhibitory factor 1
FEN1	Flap Endonuclease 1
PCNA	Proliferating cell nuclear antigen
MMR	Miss Match Repair
BER	Base Excision Repair
NER	Nucleotide Excision Repair
CPT	Camptothecin
53BP1	p53-Binding Protein 1
RUVBL1	RuvB-like 1
RUVBL2	RuvB-like 2
TDP1	Tyrosyl-DNA Phosphodiesterase 1
HAT	Histone acetyltransferases
XCCR	X-ray repair cross-complementing protein 1
PKC	Protein kinase C
BCR	B cell Receptor
AML	Acute myeloid leukemia
PI3K	Phosphotidyinositol 3 kinase
ABC and GCB	Activated B-cell and germinal center B-cell
CDC73	Cell division cycle 73
PAFc	Polymerase-associated factor complex
HDAC3	Histone deacetylase 3
CHIP	Carboxyl terminus of Hsp70-interacting protein
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1
IGF2BP3	Insulin-like growth factor 2 mRNA-binding proteins 3
LINC01138	long intergenic non-protein coding RNA 1138
CCNE1	Cyclin E1
NHERF2	Sodium-hydrogen exchange regulatory cofactor
PDZ	PSD-95, Disk-large, ZO-1
RIOK1	RIO Kinase 1
pICln	Ion Chloride nucleotide-sensitive protein
JAK2	Janus Kinase 2
TRAF-6	TNF Receptor Associated Factor 6
MTA	Methylthioadenosine
MTAP	Methylthioadenosine phosphorylase
NHL	Non-Hodgkin lymphoma
BRCA1	Breast cancer susceptibility protein 2

MLLr	Mixed-lineage-leukemia rearranged
STAT1	Signal Transducer and Activator Of Transcription 1
PD1	Programmed cell death protein 1
ULK1	Unc-51-like autophagy-activating kinases 1
ULK2	Unc-51-like autophagy-activating kinases 1
PIK3C3	phosphatidylinositol 3-kinase catalytic subunit type 3
VPS34	vacuolar protein sorting 34
FIP200	FAK family kinase-interacting protein of 200 kDa
PIK3R4	Phosphoinositide-3-kinase regulatory subunit 4
VPS15	Vacuolar protein sorting 15
PtdIns3P	Phosphatidylinositol 3-phosphate
NRBF2	Nuclear receptor binding factor 2
UVRAG	UV radiation resistance associated gene
SH3GLB1	SH3 Domain Containing GRB2 Like, Endophilin B1
RUBCN	Rubicon autophagy regulator
WIPI	WD repeat protein interacting with phosphoinositides
P62/SQSTM1	Sequestosome 1
NBR1	Neighbor of Brca1
TAXIBP1	Tax1 binding protein 1
NDP52	Nuclear dot protein 52kDa
OPTN	Optineurin
AMPK	AMP-activated protein kinase
ATF4	Activating transcription factor 4
NEDD4L	NEDD4 like E3 ubiquitin protein ligase
PKCα	Protein kinase C alpha
MBP	Myelin Basic Protein
PDK1	Pyruvate Dehydrogenase Kinase 1
TBK1	TANK-binding kinase 1
ERBB2	Erb-b2 receptor tyrosine kinase 2
NHEJ	non-homologous end joining
TDRD3	Tudor domain containing 3
WDR5	WD repeat-containing protein 5
NFIB	Nuclear factor I B
SMNDC1	Survival motor neuron domain containing 1
SND1	Staphylococcal nuclease domain-containing protein
USP9X	Ubiquitin specific peptidase 9 X-linked

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Abstract

PRMT5 (Protein arginine methyltransferase 5) is the predominant type II PRMT that monomethylates and symmetrically dimethylates arginine residues of histone and none-histone proteins to regulate diverse cellular processes. PRMT5 overexpression has been implicated in tumorigenesis and other diseases and has gained traction as a potential antitumor target with some of its inhibitors being tested in clinical trials. Despite the well-established antitumor effect of PRMT5 inhibitors, how the efficacy of these inhibitors is regulated is unexplored. We show in this study that autophagy blockage enhances cellular sensitivity to PRMT5 inhibitor in triple negative breast cancer cells. Both genetic depletion and pharmacological inhibition of PRMT5 evoke cytoprotective autophagy. We further establish that PRMT5 suppresses basal autophagy across different breast cancer types. Mechanistically, PRMT5 catalyzes monomethylation of ULK1 at R532 to suppress its autophagic functions. As a result, ULK1 inhibition or deletion blocks PRMT5 deficiency-induced autophagy and sensitizes cells to PRMT5 inhibitor. Our study identifies inducible autophagy as an important determinant of cellular sensitivity to PRMT5 inhibitor, and also establishes ULK1 as a bonafide substrate of PRMT5 in the autophagy, providing a rationale for combining PRMT5 and autophagy inhibitors in cancer therapy.

Central Hypothesis

Arginine methylation is a common posttranslational modification that has gained great attraction in the past decade due to its critical roles on diverse cellular processes. Among the nine PRMTs, PRMT5 is the dominant type II enzyme, which generates most of the SDMA on diverse nuclear and cytoplasmic substrates to control many critical cellular processes including transcription, DNA damage repair and signal transduction[1]. Overexpression of PRMT5 has been observed in various types of human cancers[2]. Notably, PRMT5 is overexpressed in more than 50% of primary breast tumors and 70% of metastatic breast tumors, with strongest expression in triple negative type breast cancer (TNBC). Moreover, elevated expression of PRMT5 is also associated with poor prognosis and survival in breast cancer patients[3,4]. Thus, PRMT5 is emerging as an attractive target for breast cancer therapy. Consequently, several PRMT5 inhibitors have been developed, with approximately eight undergoing different phases of clinical trials, including a phase II trial focused on early-stage breast cancers[5]. Preclinical studies has shown that compared to two-day treatment for most inhibitors, more than four-day treatment is required for PRMT5 inhibitor to achieve anti-proliferative effect in most cancer cells[6], indicating that a resistant mechanism may be induced at the early stage of treatment. Interestingly, manifold studies have demonstrated that autophagy induction plays a key role in modulating drug response, efficacy, and resistance in various cancers including in TNBC[7,8]. We, therefore, hypothesized that inhibition or depletion of PRMT5 induces cytoprotective autophagy in TNBC that leads to resistance to PRMT5 inhibitors. Two specific Aims are proposed (**Figure A**).

Aim 1: Determine if PRMT5 inhibitor synergizes with autophagy inhibitors to suppress TNBC growth.

Autophagy is a homeostatic and survival mechanism that is induced by stresses such as starvation and targeted therapies. It is generally believed that autophagy is crucial to drug resistance in cancer treatments[7,8]. Thus, we hypothesized that PRMT5 inhibition induces autophagy and confers resistance to PRMT5 inhibitors. This aim will determine the IC50 of various breast cancer cells to PRMT5 inhibitor, and thereby reiterate sensitive and resistant cell lines. We will further determine the minimal cytotoxic concentration of chloroquine (CQ) to TNBC. Finally, we will determine how blocking autophagy with CQ will increase sensitivity of TNBC to PRMT5 inhibitor.

Aim 2: Elucidate the molecular mechanism by which PRMT5 regulates autophagy through ULK1.

PRMT5 methylates cytoplasmic and nuclear substrates, including AKT1, GLI1, RUVBL1, and histones that are involved in transcription, signal transduction, cell cycle, and DNA damage repair[9,10,11,12]. Though the role of PRMT5 in autophagy has not been documented, one study showed that PRMT5 binds to ULK1[13]. However, the role of this binding remains unknown, which will be addressed in this Aim. Specifically, we will establish that PRMT5 methylates ULK1 to regulate its kinase activity and autophagy, as well as determine if ULK1 inhibitor sensitizes TNBC cells to PRMT5 inhibitor.

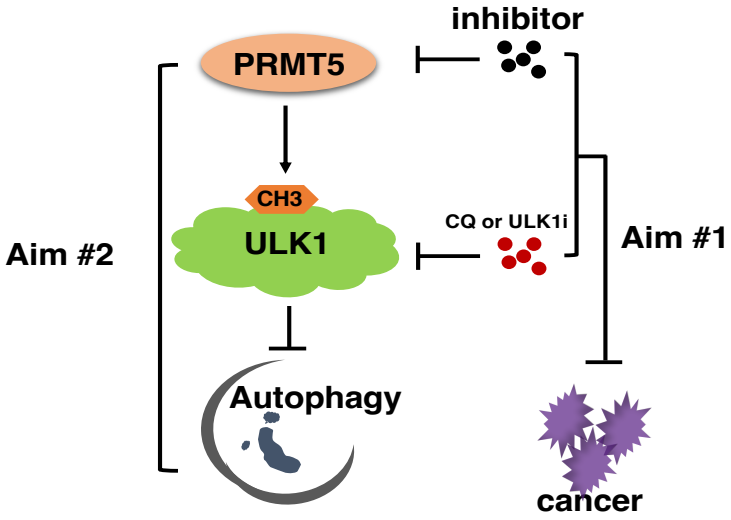


Figure A. Schematic model of proposed Aims. Aim 1 will investigate whether blockage of autophagy using chloroquine (CQ) or ULK1 inhibitor (ULKi) sensitizes breast cancer cells to autophagy. Aim 2 will interrogate the mechanism by which PRMT5 regulates autophagy through methylating ULK1.

Chapter 1: Introduction

1.1 Arginine Methylation

In addition to alternate splicing, posttranslational modifications (PTMs) are the major contributors to proteome diversity. PTMs entail enzymatic and non-enzymatic addition of chemical groups to certain amino acid residues[14], such as phosphorylation, acetylation, glycosylation, ubiquitylation, lipidation, sumoylation, biotinylation, nitrosylation, and methylation that affect many biological processes from proliferation to apoptosis[15-17]. Proteomic studies have revealed that arginine methylation is at par with serine phosphorylation and lysine ubiquitylation as the most prevalent PTMs[18]. Arginine methylation in mammal is executed by a family of nine enzymes called protein arginine methyl transferases (PRMTs), which add methyl groups on the terminal guanidino nitrogen atom using S adenosyl methionine (SAM) to produce three types of methylated arginine namely: monomethylarginine (MMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). The addition of methyl groups to arginine residues does not alter the net charge of the molecule, but affects its shape, hydrogen bonding capacity, and protein interactions, thereby influencing their biochemical functions.

1.2 Writers of Arginine Methylation

There are nine members of PRMTs in mammal categorized into three types based on the methylation type catalyzed (**Figure 1.1**).

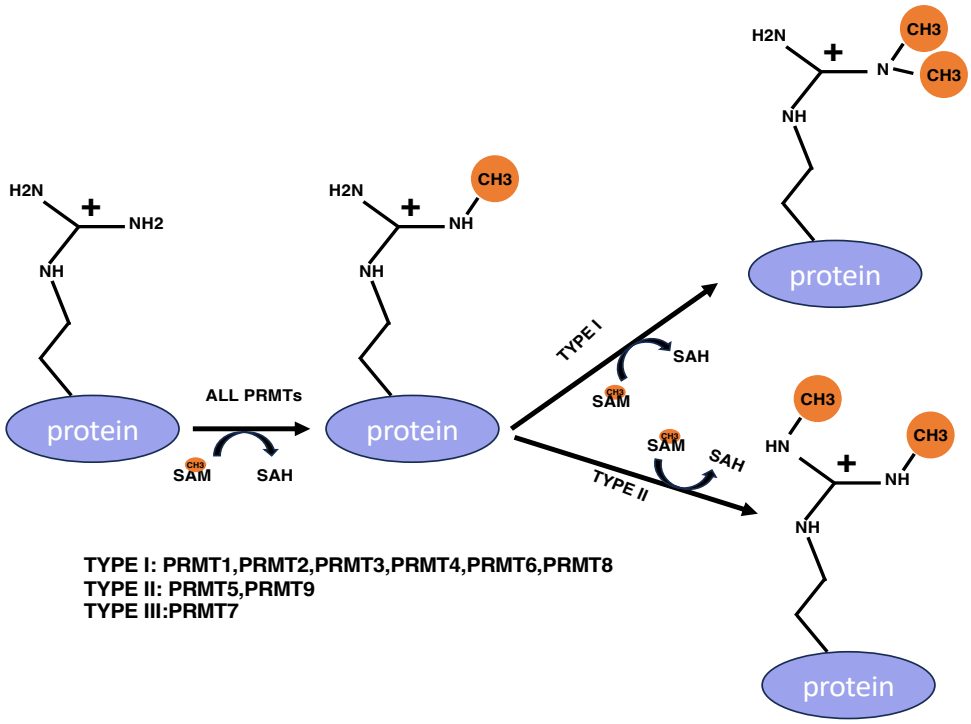


Figure 1.1. Types of PRMTs and arginine methylation. Type I, II, and III PRMTs generate monomethylarginine (MMA) as a first step, followed by asymmetric dimethylarginine (ADMA) by type I and symmetric dimethylarginine (SDMA) by type II on the terminal nitrogen atoms of the guanidino group using S adenosylmethionine (SAM).

Type I PRMTs which generate MMA and ADMA include PRMT1, PRMT2, PRMT3, PRMT4/CARM1, PRMT6 and PRMT8, while Type II PRMTs catalyze MMA and SDMA reactions and consist of PRMT5 and PRMT9. PRMT7 is the only known type III PRMT that generates only MMA[19,20], and no known PRMT possesses both SDMA and ADMA enzymatic capabilities. PRMTs are ubiquitously expressed across all tissues of the body except PRMT8 which is neuron restricted[21,22]. The generation of PRMT- specific knockout mice has expounded the critical biological roles of PRMTs from embryogenesis to adulthood, highlighting the versatility of these enzymes. Indeed, the loss of PRMT1 and PRMT5, which are predominant type I and type II PRMTs, respectively, is embryonic lethal in mice[23,24]. Apart from PRMT8 which has mutations-associated cancers[25,26], overexpression of most PRMTs has been associated with poor prognosis of cancers (**Table 1.1**)[12,27-47].

The core crystal structure of PRMT3 was the first PRMT structure to be solved using protein crystallography over two decades ago[48], after which structure of other PRMTs became established. Generally, all PRMTs possess a conserved catalytic core composed of Rossman fold where SAM binds and β-barrel which assists in substrate binding (**Figure 1.2**)[49]. The N-terminus is the most variable regions of the PRMTs that has been speculated to contribute to variation in substrate specificity and regulation[20,50]. How and what specific arginine residues are methylated by PRMTs is still subject of debate. However, available data and evidence suggest that PRMTs usually methylate arginine residues in glycine-rich (RGG/GAR) motifs of histone and non-histone proteins.

Table 1. PRMTs in cancers

PRMT	Associated Cancer	References
PRMT1	Breast, lung, hematological malignancies, colon, prostate	29 - 35
PRMT2	Breast	36
PRMT3	Breast	37
CARM1/PRMT4	Breast, prostate, colorectal	37 - 39
PRMT5	Lung, hematological malignancies, melanoma, gastric, colorectal, breast	39 - 43
PRMT6	Bladder, lung, and hepatocellular carcinoma	44,45
PRMT7	Breast cancer	44, 46
PRMT8	Ovarian, skin, gastric	25
PRMT9	Hepatocellular carcinoma	47

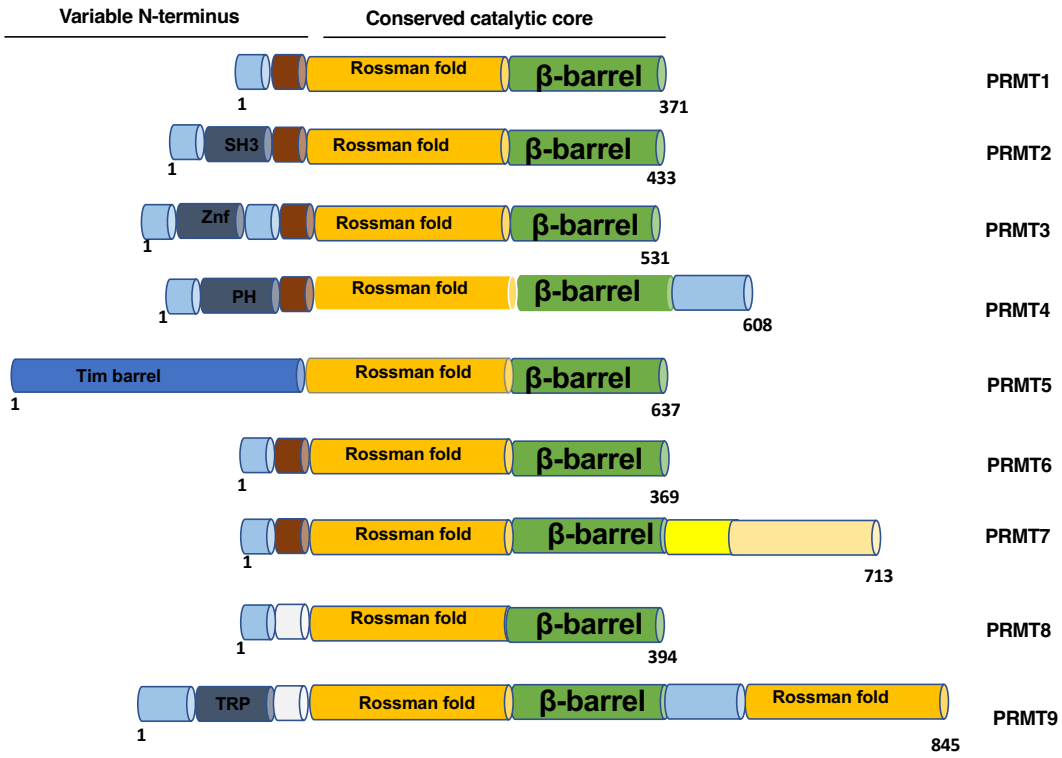


Figure 1.2. Schematic structure of PRMTs. All PRMTs despite their varying length possess catalytic core made up of Rossman fold where SAM binds and β-barrel which assist in substrate binding, and variable N-terminus.

However, not all PRMTs follow this rule: PRMT4 can methylate arginine surrounded by proline, glycine, and methionine (PGM), while PRMT7 prefers arginine (RXR) embedded in lysine-rich environment[12,51]. Despite substrate specificities of PRMTs, the fact that global SDMA and ADMA increase when PRMT1 and PRMT5 are inhibited respectively, suggests substrates sharing among PRMTs[52,53].

1.3 Readers of Arginine Methylation

Methylated arginines serve as docking sites which are recognized by certain proteins for protein-protein interactions. Most of these readers of methylated arginine possess Tudor domains that forms an aromatic cage responsible for recognizing methylated arginines. While there are an estimated 40 Tudor domain proteins, few have been identified as methylated arginine effectors, with SMN and TRD3 being the most characterized. Interestingly, Tudor domain proteins like SMN, SMNDC1, and SND1 have a relatively high affinity for SDMA than ADMA.

SMN is involved in important cellular processes like splicing, transcription, and translation[54]. Blocking of arginine methylation impairs ability of SMN Tudor domains to interact with substrates that negatively affects certain biological process. For example, arginine methylation-deficient RNA Polymerase II blocks the SMN-mediated resolution of R-loops[55]. SMNDC1 also known as SPF30 is partially identical to SMN but binds mildly to methylated arginine. It associates with RNA binding proteins in regulating splicing, ribosome biogenesis and chromatin remodeling[56,57]. Similarly, SND1 or p100/TSN effector functions are involved in RNA interference. Hu et al. showed that SND1 binds to PRMT5-

mediated methylated RISC component, AGO2, to promote degradation of AGO2- linked sRNAs[58]. Similarly, SND1's effector function is involved in controlling alternate splicing of E2F-1 target genes[59,60]. TRD3 appears to be the only Tudor domain protein that has a preferentially high affinity for ADMA. Its effector functions are vital for TOP3B-mediated R-loop resolution and USP9X ubiquitylating activity[61,62].

There are other methylated arginine readers that are devoid of Tudor domains, most of which have affinity for ADMA than MMA or SDMA. These proteins were identified through their ability to bind methylated arginine residues of histone and non-histone proteins, and in most cases, these bindings were abrogated or reduced upon deletion of the PRMT responsible for the methylation or mutating the arginine residues. For example, methylation of p300 by PRMT4 promotes its interaction with BRCA1[63], while methylation of chromatin remodeler Potin by PRMT4 enhance its binding to FOXO3a[64]. Other non-Tudor domain effectors include Brg1, an ATpase subunit of SWI/SNF complex, that binds H4R3me2a[65]; PAF1c that binds H3R17me2a[66]; TRIM29 that binds methylated NFIB[67]; and WDR5 that binds H3R2me2s[68].

1.4 Erasers of Arginine Methylation

Given that arginine methylation plays critical biological roles germane for survival and proliferation, it is plausible to assume that there should be demethylases to erase these modifications as a way of regulating their consequences. Whether there is an obligate arginine demethylase is not yet established. Nonetheless, some reports allude to impermanence of methylated arginines. The peptidyl-arginine deiminases (PADs) catalyze

the hydrolysis of peptidyl-arginine to peptidyl-citrulline. PAD4 was initially believed to be an arginine demethylase because it demethylated monomethylated arginine residues of H3 and H4[69]. However, since citrulline which is chemically different from unmethylated arginine is the product of this reaction, PAD4 is not considered a true demethylase. The Jumonji domain-containing protein 6 (JMJD6), which is 2OG-dependent JumonjiC (JmjC)-domain-containing oxygenase, also possesses some arginine demethylase properties. JMJD6 is able to demethylate methylated arginines of H3, H4[70] and non-histone proteins like estrogen receptorα[71,72], HSP70[73] and G3BP1[74]. Despite this evidence, JMJD6 has been reported to possess lysine C-5 hydroxylase activities on certain splicing regulatory proteins, casting doubt on it as an obligate arginine demethylase[75-77]. Similarly, some JmjC histone lysine demethylases (KDM) like KDM3A, KDM4E, and KDM5C also doubles as arginine demethylases for both histone and non-histone proteins[78-80]. Thus far, there is no known demethylase specifically for methylated arginine residues, warranting further studies.

Chapter 2: PRMT5 – Structure , Regulation, and Functions

2.1 Overview of PRMT5

Through a two-hybrid system, the human PRMT5 was first discovered as a JaK-2 binding protein 1 (JBP1) that has methyltransferase properties[81]. The human PRMT5 is a 637-amino-acid-long protein that possesses a triosephosphate isomerase (TIM) barrel domain, supporting the formation of the PRMT5:MEP50 octameric complex, and a β-barrel at the C-terminus required for its dimerization (**Figure 2.1**)[82,83]. It forms a hetero-octameric complex with methylosome protein 50 (MEP50, also called WDR77) of about 450 KD for maximum enzymatic activity[84]. Evidence of the critical role of MEP50 towards PRMT5 enzymatic function was reported when PRMT5 purified from bacteria was not able to methylate certain substrates, highlighting the indispensability of MEP50 for PRMT5 maximum enzymatic function[85]. As the dominant type II PRMT, the biological functions of PRMT5 are dictated by its substrates involved in cellular processes like DNA damage response, proliferation, cell cycle, apoptosis, transcription, splicing, and cell signaling. Over the last two decades, a preponderance of data has implicated PRMT5 as a major driver of several cancer types. This has necessitated the use of various tissue-specific knockout mice and *in vitro* knockout models to elucidate its tumorigenic functions[86].

PRMT5 executes MMA and SDMA reactions that could occur in processive or distributive manner. Distributive process will cause the release of the substrate after the first methylation event before rebinding for the second MMA to be added, while in processive reaction, both MMA and SDMA occurs simultaneously before the substrate is released[82,87].

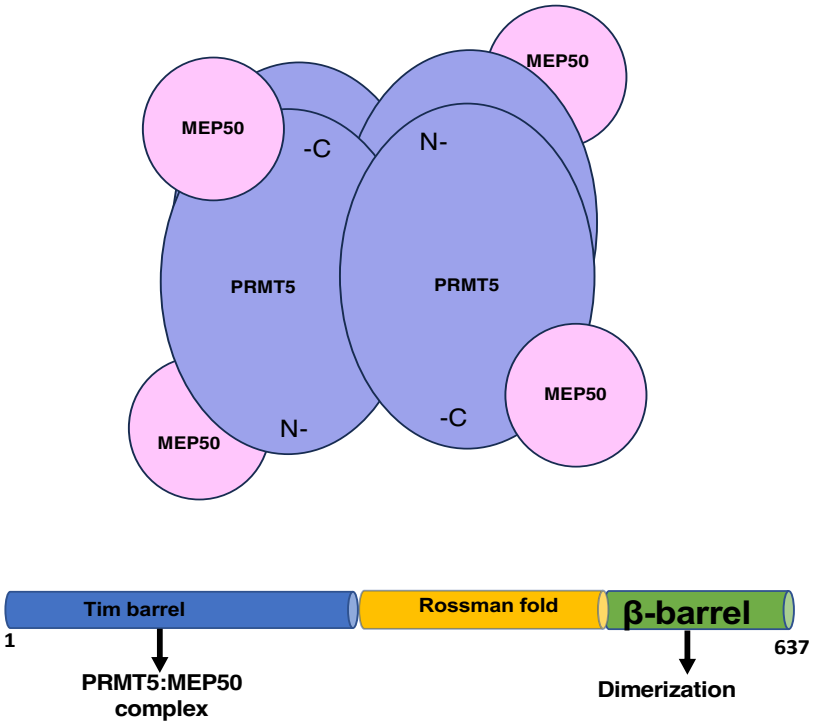


Figure 2.1. Structure of the human PRMT5-MEP50 hetero-octameric complex. Pictorial view of the subunit arrangement of the hetero-octameric PRMT5–MEP50 structure with the head-to-tail N-terminal and C-terminal PRMT5 arrangement shown by ‘‘N-’’ and ‘‘-C’’, respectively. Below is a representation of domains of PRMT5 responsible for MEP50 binding and dimerization.

2.2 Regulation of PRMT5

Oncogenic activities of PRMT5 are mostly associated with its overexpression because evidence of mutation, duplication or deletion are scanty. However, transcriptional, and post transcriptional control of PRMT5 functions have been documented. Broadly, PRMT5 activities are regulated through its transcription, protein stability, localization, and enzymatic activity.

2.2.1 Transcriptional Regulation of PRMT5 Expression

Certain transcriptional factors modulate PRMT5 expression across different cell types. In prostate cancer cells, PKC activation regulates PRMT5 expression through NF-Y transcription factor. NF-Y binds two identical CAATT repeats at the proximal promoter region to drive PRMT5 transcription[88]. PKC activation upregulates c-Fos which suppresses NF-Y, thus, suppressing PRMT5 transcription. PRMT5 expression is also stimulated in response to BCR activation. Specifically, BCR activation promotes PRMT5 transcription via TBK-NF-κB in DLBCL cells and via PI3K/AKT/GSK-3β/MYC-pathway in ABC and GCB DLBCL cells[89]. The CDC73 subunits of PAFc recruits other transcriptional activators to PRMT5 promoters to drive its expression in leukemic cells[90]. Similarly, PRMT5 expression is regulated via NF-κB-HDAC3 pathway that suppresses miR96 which typically downregulates PRMT5 expression especially in transformed B cells[91] . PRMT5 was shown to promote BCR-ABL fusion gene transcription. Simultaneously, BCR-ABL also facilitated the transcriptional upregulation of PRMT5 by recruiting STAT5 and STAT5B to the PRMT5 promoter, suggesting a potential positive feedback regulation[92].

2.2.2 Regulation of PRMT5 Protein Stability

The E3 ligase, CHIP, promotes PRMT5 degradation through K48-linked ubiquitylation. It is speculated that HSP90 could serve as a regulatory chaperone for proper folding of the PRMT5 protein, as evidenced by increased degradation of PRMT5 upon HSP90 inhibition by CHIP[93]. Thus, PRMT5 might be one of the downstream players of HS90 inhibitors-mediated cell death. The aforementioned pathway is regulated upstream of the IGF2BP1/IGF2BP3/LINC01138 pathway, where IGF2BP1/IGF2BP3 promote the stability of lincRNA LINC01138. This lincRNA interacts with PRMT5 to shield it from CHIP-mediated degradation in liver cancer cells[94].

2.2.3 Regulation of PRMT5 Localization and Substrate Specificity

PRMT5 localizes in cytoplasm, nucleus, and cell membrane. Most of this localization is dictated by its binding partners, which sometimes confer some specificities. RIOK1 and pICln compete for binding to the N-terminus of PRMT5. RIOK1 recruits nucleolin, while pICln recruits sm proteins, respectively, for PRMT5-mediated methylation[95,96]. Co-operator of PRMT5 (COPR5) also binds and recruits PRMT5 to *CCNE1* promoter but not *NM23*, where PRMT5 specifically methylates histone H4 but not H3. Thus, COPR5 regulates PRMT5 substrate specificity[97]. C-terminus of PRMT5 has been shown to be phosphorylated by AKT and SGK kinases. This phosphorylation promotes its binding to 14-3-3 protein, while the unphosphorylated protein binds to PDZ domain proteins like NHERF2, suggesting that PRMT5 undergoes PDZ/14-3-3 switch. The PDZ/14-3-3 switch

recruits PRMT5 to the membrane and is critical for mouse viability. Mice with a homozygous deletion of the region responsible for this switch suffer embryonic lethality[98].

2.2.4 Regulation of PRMT5 Enzymatic Activity

The fact that recombinant PRMT5 isolated from bacteria has limited enzymatic functions, suggests other biochemical processes and/or interactions are germane for full activation of PRMT5. In addition to MEP50 which is indispensable for optimal PRMT5 activity, PTMs have been shown to influence PRMT5 enzymatic functions. Cyclin D1/CDK4 phosphorylates MEP50 to promote PRMT5 activity towards methylation of H3R8 and H4R3[99]. PRMT5 is phosphorylated at three threonine residues by LKB1 in breast cancer cells without affecting its localization and dimerization. However, mutating these phosphorylation sites decreased its methyltransferase activity as well as decreased its binding to RIOK1, pICln and MEP50[100]. Similarly, Oncogenic JAK2 mutant kinase JAK2V617F phosphorylates tyrosine residues in the N-terminus region of PRMT5. This phosphorylation hinders its binding to MEP50 to reduce its methyltransferase activity towards histone tails. Knockdown of PRMT5 promoted proliferation and differentiation of erythroid cells, suggesting this JAK2V617F oncogenic functions is executed in part through phosphorylating PRMT5 to suppress differentiation of erythroid cells[101]. In human erythroleukemia Lys-562 cells, CARM1/PRMT4 methylates PRMT5 to impair its oligomerization, suppressing its enzymatic function. Consequently, the presence of methylation-deficient PRMT5 resulted in reduced H4R3me2s on the γ-globin gene promote. Thus, CARM1 could be targeted in dealing with PRMT5 oncogenic activities in

hemoglobinopathies[102]. TRAF6 also promoted K63-linked ubiquitylation on lysine residues in N-terminal TIM barrel domain of PRMT5 that enhances its interaction with MEP50 and increase its methyltransferase activities. This modification was critical for breast cancer cells proliferation as ubiquitylation-deficient PRMT5 failed to develop xenograft tumors compared to the wild type[103].

2.3 Biological Roles of PRMT5

2.3.1 Transcriptional Regulation of Genes

PRMT5 executes its biological functions in part by symmetrically methylating arginine residues of histone tails to dictate target gene expression. Currently, H4R3, H3R2, and H3R8 are the identified histones methylated by PRMT5[12,104,105]. Generally, symmetric dimethylation of H4R3 and H3R8 (H4R3me2s and H3R8me2s) at the promoters of target genes is associated with transcription repression, especially tumor suppressors (**Table 2. 1**). PRMT5 in complex with PHF1 and CUL4B registers H4R3me2s mark on the promoters of E-cadherin and FXBW7 to repress their expression in breast cancer cells[106]. Since E-cadherin and FXBW7 have known tumor suppressor functions[107,108], their transcriptional repression by PRMT5 promotes cancer development. In leukemic cells, PRMT5 methylates H3R8 and H4R3 on the promoters of *ST7*, *NM23* , *RBL1, RB1* and *RBL2* to negatively regulate their transcription[109-111]. MicroRNAs that generally repress proliferative genes are also bonafide substrates of PRMT5. Promoters of miR33b, miR96, and miR503 are methylated on H3R8 to repress their expression, leading to increased Cyclin D1 and c-MYC expression in NHL[112], while miR99 and miR2b are methylated on H4R3 to downregulate

their expression to promote FGFR and FLT3 expression in lung cancer and AML[113,114]. Gurung et al. reported that Menin recruits PRMT5 to *GLI1* and *Gas1* promoters where PRMT5 methylates H4R3, leading to repression of *GLI1*, *Ptch1*, and *c-Myc* as well as hedgehog (Hh) signaling[10,11]. Thus, mutations in Menin, which abrogate its binding to PRMT5, lead to uncontrolled Hh signaling to drive tumorigenesis. The E3 ligase CUL4A/B, which targets the replication licensing protein CDT1 for degradation to regulate cell growth, is repressed through PRMT5-dependent methylation of H3R8 and H4R3. This methylation event stabilizes CDT1 to drive tumorigenesis[99]. Another important transcriptional role of PRMT5 in organismal development is evidenced in its ability to restrict fetal globin expression in primates. As primates grow from birth to adulthood, fetal globin is repressed in adult bone marrow. Zhao et al. showed that PRMT5-guided methylation on fetal globin promotes recruitment of DNMT3A, which methylates CpG islands to suppress fetal globin transcription and expression in adult bone marrow[115].

Not always does methylation of histones by PRMT5 results in transcription repression of target genes, it also upregulates target gene expression. In colorectal cancer, FGFR3 and eIF4E are transcriptionally upregulated when methylated by PRMT5 at their promoters[116]. H4R3 of androgen receptor (AR) is dimethylated when PRMT5 is recruited by Sp1 and Brg1 complex to its proximal promoter to enhance AR and its target expression to advance prostate cancer[117]. Cao et al. revealed that PRMT5 in complex with JDP2/β-catenin methylates H3R2 of *SLC7A11, GSS*, and GCLM promoters, leading to activation of glutathione metabolism to circumvent stress and allow ovarian cancer cells to thrive[118]. PRMT5 also enhances the expression of RNF168, an E3-ubiquitin ligase that regulates

H2AX during DNA damage repair. This is achieved by depositing H3R2me1 (monomethylation) on its promoter, facilitating the ubiquitination and stabilization of H2AX, which is crucial for genome integrity in glioblastoma models[119]. In lung cancer, TGF-β treatment induces PRMT5-mediated deposition of H3R2me1, facilitating MLL-WDR5-mediated upregulation of EMT genes such as *SNAIL* and downregulation of anti-EMT genes, ultimately promoting metastasis[120].

PRMT5 also affects transcription by directly methylating non-histone proteins (**Table 2.2**). PRMT5 dimethylates p65 (RelA) subunit of NF-κB upon IL-1β exposure to promote NF-κB DNA binding to activate target genes[121]. Interestingly, subsequent study of PRMT regulation of NF-κB signaling revealed that PRMT1 methylates the same arginine residue, but with a different outcome: PRMT1-mediated methylation of p65(RelA) compromised its DNA binding capacity, resulting in the repression of NF-κB target genes in response to TNFα[122]. Thus, in response to variable stimuli, NF-κB vacillates between SDMA and ADMA to regulate its transcriptional functions.

The tumor suppressor p53 is methylated on arginine residues within its putative oligomerization domain, which plays a crucial role in dictating its transcriptional activity. Known p53 target genes like *GADD45* and *APAF-1* were activated by arginine methylation-deficient p53, while not affecting p21, suggesting a gene-specific regulation of p53's transcriptional activities by PRMT5[123]. Another transcription factor E2F-1, which has both oncogenic and tumor suppressor functions[124], is methylated by PRMT5. This methylation promotes E2F-1 degradation to drive a reduction in transcription output of E2F-1 target genes like apoptotic p73 gene. It is believed that high expression of PRMT5 in cancer cells

keeps low levels of E2F-1 to allow cancer cells to evade prevalent DNA damage-induced apoptosis[125]. An important cell-fate decision regulator, KLF4, is also methylated by PRMT5 to prevent its ubiquitylation and degradation by VHL/VBC E3 ligase. In breast cancer, stabilized KLF4 upregulates p21 and suppresses the pro-apoptotic factor BAX, enabling cancer cells to tolerate DNA damage-induced cytotoxicity[126]. Similarly, SREBP1a stability is enhanced via PRMT5-mediated methylation. Mechanistically, this methylation inhibits GSK3β-mediated phosphorylation, preventing SREBP1a from binding to the Fbw7 ubiquitin ligase for degradation. The stabilized SREBP1a then activates lipogenic target genes, promoting the progression of hepatocellular cancer[127]. In prostate cancer characterized by the overexpression of the ETS transcription factor ERG, PRMT5 is recruited by ERG to methylate the ligand binding domain of the androgen receptor (AR). This process results in reduced AR receptor recruitment and diminished induction of target genes such as PSA, which plays a role in the differentiation of prostate epithelium. Consequently, this alteration grants stemness-like features to cancer cells[128].

Additionally, in melanoma, the TGF-β pathway is antagonized through SHARPIN-PRMT5-guided methylation of SKI, a component of the TGF-β pathway. This methylation inhibits SKI's repressor function, as indicated by the increased expression of SOX10 and MITF transcription factors, ultimately contributing to melanoma tumorigenesis[129]. In the context of BCL6, a transcriptional repressor involved in repressing genes responsible for lymphocyte differentiation and inflammation, its repressive functions are enhanced by PRMT5-mediated methylation. This methylation plays a critical role in BCL6-dependent germinal center formation, efficient immunoglobulin production, and contributes to the

severity of diffuse large B-cell lymphoma (DLBCL). The overexpression of PRMT5 further leads to uncontrolled proliferation of transformed lymphocytes[130]. In a similar study interrogating the role of PRMT5 in T cell biology, conditional knockout mice with PRMT5 deficiency in Tregs presented with scurvy like autoimmunity with excessive lymphocyte invasion of the liver. In demystifying this phenotype, the transcription factor FOXP3, crucial for regulatory T cell (Treg) development and function, was found to be symmetrically methylated by PRMT5. The methylation of FOXP3 was essential for Treg suppressive function, particularly in peripheral lymph nodes, where Tregs lacking arginine methylation displayed reduced effector T cell function[131]. Thus, the autoimmunity observed in mice with PRMT5 depletion in Tregs was in part through impaired Foxp3 methylation.

Summarily, PRMT5 overexpression in many cancers enhances its capacity to methylate histone residues, modifying chromatin structure and accessibility to transcriptionally suppress or activate specific genes. It can also directly methylate transcriptional activators or repressors, influencing their stability or binding capacity to regulate their transcriptional output, ultimately promoting tumor growth.

Table 2.1. PRMT5 histone substrates and associated cancers

Substrate	Target protein	Transcriptional ramification	Cancer type	References
H4R3	GLI1, Ptch1, and c-Myc	Repression	Endocrine tumor	12,13
H3R8, H4R3	ST7, NM23 and RBL2, RBL1, and RB1	Repression	leukemia	109, 110
H4R3	CUL4A/B	Repression	Lymphoma	99
H4R3	E-cadherin, *FXBW7*	Repression	Breast	106
H3R8, H4R3	miR33b, miR96, miR503, miR99 and miR2b	Repression	Lymphoma, lung, AML	112,113,114
H4R3, H3R8	FGFR3, eIF4E	Activation	Colorectal	116
H4R3	androgen receptor (AR)	Activation	Prostate	117
H3R2	SLCA7A11/GSS and GCLM, RNF168	Activation	Ovarian, GBM	118,119

Table 2.2. PRMT5 non-histone substrates and associated cancers

Substrate	Ramification	Cancer type	References
p65(RelA)	Methylation activate NF-kB transcriptional activities	colorectal	121
P53	Dictates p53 transcriptional activity	Sarcoma, lymphoma	123
E2F1	Promotes E2F1 degradation to promote growth	Colon	124
KLF4	Stabilizes KLF4 which increased transcription of p21 and represses BAX protein	Breast	126
SREBP1a	Stabilizes SREBP1 to increase lipogenesis	Hepatocellular carcinoma	127
Androgen receptor (AR)	Represses AR recruitment to target genes promoters	Prostate	128
SKI	Inhibits SKI repressor functions	Melanoma	129
BCL6	Promotes BCL6 repressive activities	Lymphoma	131
sm proteins: SmB, SmD1 and SmD3	Facilitates their loading on the snRNA and subsequent formation of the U snRNP	Unknown	137
SRSF1	Promotes SRF1 interaction with mRNA and splicing proteins to regulate splicing	AML	139
ZNF326	proper mRNA splicing at A-T rich regions	Breast	140
RPS10	Enhance RPS10 loading unto ribosomes to accelerate translation	Hepatoma	143
RAF proteins: CRAF and BRAF	Methylation destabilized RAF proteins to dampen ERK activation	Pheochromocytoma	146
PDGFRα	Increases stability	Oligodendroglioma	147
EGFR	Decreases EGFR-ERK signaling	Breast	149
GLI1	Increases GLI1 half life	Hh expressing tumors	150
FEN1	FEN1 methylation enhance its interaction with PCNA that facilitates its recruitment to replication and repair foci to remove flap structures	unknown	152
53BP1	Stabilizes 53BP1 to promote DSB	Unknown	153
RAD9	Enhance its DDR activities	unknown	156
RUVBL1	Enhance DSB repair by augmenting TIP60 activities	Unknown	161
TDP1	Enhance its enzymatic functions	unknown	165

2.3.2 Splicing

The presence of glycine-arginine-rich (GAR) motifs in many RNA-binding proteins makes them potential substrates for protein arginine methyltransferases (PRMTs). This connection between arginine methylation and RNA metabolic processes, such as splicing, highlights the intricate role of PRMTs in regulating various aspects of RNA biology[132]. The first indirect evidence implicating PRMT5 in mRNA splicing was recorded when pan-SDMA inhibitor blocked the splicing of adenovirus major late (AdML) pre-mRNA *in vitro*[133]. Subsequent studies have expanded our knowledge of PRMT5 substrates in splicing and how PRMT5 in concert with these substrates promotes cancer development. Splicing is executed by the spliceosome complex which is composite of five small nuclear ribonucleoprotein particle (snRNP) and other non-snRNP proteins. Each snRNP is made up of short noncoding RNA (snRNA), ring of seven sm proteins and other individual snRNP-specific proteins[134]. Newly synthesized small nuclear RNAs (snRNAs) are exported into the cytoplasm, where they associate with the SMN complex. This complex recruits small nuclear ribonucleoprotein (snRNP) proteins to form the core snRNA, which is then shuttled back into the nucleus. In the nucleus, the core snRNA traffics through Cajal bodies before accumulating in the nucleoplasm, where the completion of snRNP biogenesis occurs. This process involves the addition of snRNP-specific proteins and snRNA modifications, culminating in the formation of a functional spliceosome[135,136]. It was revealed through *in vitro* studies that PRMT5-mediated methylation of the sm proteins (SmB, SmD1, and SmD3) is required for their loading on the snRNA and subsequent formation of the U snRNP[137]. Indeed, Bezzi et al. through the conditional knockout of

PRMT5 in the central nervous system of mice, later showed that sm proteins were hypomethylated. This hypomethylation resulted in intron retention and exon skipping in genes with weak 5' donor sites, indicative of impaired splicing. This PRMT5 knockout study also established that *mdm4* which inhibits p53 activities undergoes exon skipping leading to expression of inactive short form (*mdm4s*)[138] . This may have implications for the regulation of p53 and other cellular processes controlled by the mdm4-p53 axis. PRMT5 also methylates SRSF1, a member of the serine arginine rich family of transcription factors, to promote its interaction with mRNA and splicing proteins to regulate splicing, a process critical for AML cells survival and proliferation[139]. Consequently, arginine methylation deficient SRSF1 mutant was cytotoxic to AML cells, suggesting that cell death upon PRMT5 depletion was partially due to impaired splicing caused by aberrant SRSF1 methylation. Similarly, ZNF326 undergoes symmetrical dimethylation by PRMT5 to facilitate proper mRNA splicing at A-T rich regions of certain genes in MDA-MB-231 breast cancer cells[140]. Likewise, PRMT5 again methylates chromatin-associated E2F-1, but this time to regulate E2F-1 splicing functions by attracting Tudor domain reader p100/TSN to accelerate alternate splicing of E2F target genes like *SENP7* and *MECOM*[59,60].

2.3.3 Translation

PRMT5 partakes in translation which is a critical cellular process central to proliferation and survival. The evolutionarily conserved nutrient-sensitive gatekeeper, mTOR, promotes translation under high-nutrient conditions by suppressing eIF4E-binding proteins (4E-BP), which inhibit eIF4E, a positive translation regulator[141]. PRMT5 at least regulated p53

translation by influencing eIF4E expression. Knockdown of PRMT5 decreased eIF4E expression which is believed to suppress p53 expression. Forced eIF4E expression was able to rescue inhibition of proliferation associated with PRMT5 depletion, indicating eIF4E plays role in PRMT5 regulation of cell proliferation[142]. However, since PRMT5 depletion have stalled cell growth irrespective of the p53 status, this might be context or cell type dependent. PRMT5 methylates RPS10, a component of the ribosomal 40S subunit complex, facilitating its assembly onto ribosomes and localization within the nucleolar GC compartment. This process enhances translation speed by influencing the stability and nuclear export of RPS10. Accordingly, translation was retarded in RS10 arginine methylation-deficient mutants in hepatoma cell lines[143]. In addition, by using IRES-dependent reporter system, Gao et al. showed that PRMT5 has a role in translation of pocket of genes with internal ribosome entry site (IRES). The heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) which has been implicated in IRES translation[144], was methylated by PRMT5 to promote its recognition and binding to IRES of cyclin D1, HIF1a, ESR1 and MYC and enhance their IRES-mediated translation[145].

2.3.4 Cell Signaling

PRMT5 plays a dual role in cell signaling pathways – directly methylating signaling proteins and indirectly influencing their transcription. This multifaceted mechanism allows PRMT5 to execute both physiological and oncogenic functions. In the context of RAS-ERK1/2 signaling, PRMT5 methylates RAF proteins (CRAF and BRAF), dampening the amplitude of ERK1/2 phosphorylation. Knockdown of PRMT5 or expression of arginine methylation-

deficient mutant RAF proteins results in sustained activation of RAS-ERK1/2 signaling upon EGF stimulation, prompting a switch in neuronal cells from proliferation to differentiation[146]. In oligodendritic cells, PRMT5 methylates PDGFRα, promoting its downstream signaling. Specifically, methylation of PDGFRα stabilizes the protein by decreasing its binding to Cbl E3 ligase, preventing subsequent degradation. Stabilized PDGFRα properly localizes to the cell membrane, a process required for oligodendrocyte differentiation and myelination. Mice with conditional depletion of PRMT5 in oligodendrocytes suffer hypomyelination and death by the third post-natal week[147]. PRMT5 also methylates EGFR at R1175, enhancing Tyr1173 phosphorylation and serving as a docking site for SH2-domain-containing protein tyrosine phosphatase 1 (SHP1). SHP1 binding reduces EGFR-mediated ERK signaling[148]. Mutating the arginine methylation sites in EGFR reduces SHP1 binding, leading to ERK activation, increased proliferation, and migration of tumor cells[149]. In Hh signaling, PRMT5 regulates transcription and protein stability of GLI1[10,11]. The PRMT5/MEP50 complex methylates GLI1 to prevent its degradation from ITCH/NUMB-mediated ubiquitylation. This allows stabilized GLI1 to translocate to the nucleus and activate its target genes in Hh signaling[150]. In this scenario PRMT5 is regulating a signaling pathway by targeting the mRNA and protein of a key player (GLI1) in the pathway. Whether this a spatiotemporal regulation warrants clarification. Similarly, PRMT5 enhance WNT/β-catenin signaling by methylating promoters of β-catenin antagonists *AXIN2* and *WIF1* to suppress their expression[92,151] .

2.3.5 DNA Damage Response

Genetic and chemical blockage of PRMT5 enzymatic activities have elicited DNA damage in both cell and mouse studies. Though the mechanism of how PRMT5 regulates DNA damage response (DDR) is not fully understood, several substrates of PRMT5 have been reported to mediate this cellular process.

During replication and DDR, FEN1 recognizes single-strand flap generated and cleaves it to create nicks which are filled by DNA ligase1. PRMT5 promotes long patch repair (LP-BER) by symmetrically dimethylating FEN1. This modification reduces FEN1 phosphorylation by cyclin E/CDK2, enhancing its interaction with PCNA. The increased interaction is believed to facilitate FEN1 loading onto flap sites, contributing to the removal of flap structures. Overexpression of arginine methylation-deficient FEN1 led to higher γH2AX signals upon DNA damage compared to wild types, making the mutant expressing cells more sensitive to oxidative stress induced DNA damage due to compromised DDR[152].

P53 binding protein 1(53BP1) is an important regulator of NHEJ DDR that accumulates at DNA damage sites to recruit certain responsive proteins. Currently, it is believed that BRCA1-dependent DDR pathways compete with 53BP1 pathways at the early stages of HR: while 53BP1-dependent pathways suppress end resection, BRCA1-dependent pathways promote it [153]. The scavenging of arginine substrates by PRMT1 and PRMT5 becomes evident in the methylation of 53BP1. Knockdown of PRMT5 decreases SDMA but increases ADMA, while the opposite is observed in PRMT1 depletion. PRMT5-mediated SDMA stabilizes 53BP1 to promote DNA double-strand break (DSB) repair. Cells

with arginine methylation-deficient 53BP1 exhibited aberrant non-homologous end joining (NHEJ) and homologous recombination (HR)-mediated repair, as evidenced by higher γH2AX levels compared to control cells[154]. PRMT1-mediated ADMA of 53BP1 on the contrary facilitates its DNA binding functions[155]. Thus, PRMT1 and PRMT5 act in concert to modulate DSB repairs.

RAD9 is a highly complex protein with two major domains: the N-terminal part that is responsible for binding Hus1 and Rad1 to form the 9-1-1 complex, and C-terminal domain that is critical for full activation of DNA damage response[156]. Rad9 plays roles in DNA damage response starting from checkpoint activation in response to replication stress and various forms of DDR such as BER, NER, and MMR. PRMT5-guided methylation of RAD9 increases in response to hydroxyurea, but it does not affect 9-1-1 complex formation. *Rad9* knockout mouse embryonic stem cells expressing methylation-compromised Rad9 mutant were more sensitive to hydroxyurea induced DNA damage compared to Rad9 wild type reconstituted cells[157].

RUVBL1 and RUVBL2 are multifunctional AAA+ ATPases that play integral roles in the INO8 family and the TIP60 histone acetylase complex[158,159]. The TIP60 acetyltransferase complex regulates the retention of 53BP1 at sites of double-strand breaks (DSBs) in homologous recombination repair. This regulation is achieved through TIP60-mediated acetylation of histone H4K16, which impedes the binding of the Tudor domain of 53BP1 to methylated H4K20[160]. PRMT5 dimethylated RUVBL1 to enhance DSB repair. Accordingly, wild type RUBVL1 but not the arginine methylation-deficient mutant was able to rescue impaired DSB caused by depletion of RUBVL1. Mechanistically, RUBVL1

methylation was required for TIP60 to displace 53BP1 from the damage site. Interestingly, both wild type and methylation-deficient RUVBL1 were effectively recruited to DSB ends, suggesting that arginine methylation does not regulate TIP60 DNA binding activities[161].

TDP1 (Tyrosyl-DNA Phosphodiesterase 1) plays a crucial role in the removal of Topoisomerase 1 cleavage complexes (Top1cc). These complexes are transient single-strand breaks formed during processes such as DNA replication, transcription, and chromatin remodeling, where Topoisomerase 1 (TOP1) is involved in relaxing DNA supercoiling. TDP1 acts to resolve these Top1cc structures, ensuring the proper functioning of cellular processes that involve DNA dynamics. When chemotherapeutic agents like Camptothecin (CPT) trap Topoisomerase 1 cleavage complexes (Top1cc), collisions with replication or transcription forks can lead to the formation of double-strand breaks (DSBs). [162-164]. TDP1 has N-terminus domain that is critical for its recruitment and stability, and a C-terminus catalytic domain which catalyzes the hydrolysis of the phosphodiester bond between Top1 and the 3' end of exposed DNA ends. PRMT5 symmetrically dimethylated N-terminus of TDP1 with the methylation increasing upon CPT treatment. The methylation of TDP1 activated its ability to repair double-strand breaks (DSBs) induced by Top1cc during replication and transcription. In this context, arginine methylation-deficient mutants of TDP1 were unable to interact with XCCR, resulting in heightened DNA damage in response to CPT treatment[165].

2.4 PRMT5 Inhibitors

The involvement of PRMT5 in tumorigenesis, prognosis, and patient survival has positioned it as an appealing target for cancer treatment, driving the need for the development of small molecule inhibitors. The inhibitors are broadly categorized into three based on the mode of action.

2.4.1 SAM Uncompetitive Inhibitors

This class of inhibitors blocks PRMT5 methyltransferase activity by occupying substrate binding site or competing with the substrate for its binding to PRMT5. EPZ015666 (GSK3235025) is the first orally bioavailable SAM uncompetitive inhibitor that antagonizes PRMT5 activity by occupying the substrate binding site of PRMT5 and interacting with critical residues, such as Glu 444, which is essential for PRMT5 enzymatic activity. It was identified by screening a library of about 370,000 small molecules against monomethylation of H4R3 by PRMT5/MEP50 on histone H4 peptide. The hit compound from this screen underwent multiparametric optimization to yield EPZ015666 and EPZ015866 (GSK3203591). These compounds exhibited relatively high anti-PRMT5 activities *in vitro*[166]. EPZ01566 was able to suppress tumor growth in mantle cell leukemia xenograft models. The binding of EPZ015666 to PRMT5-MEP50 was high when SAM is bound to PRMT5/MEP50, suggesting a dependence of this inhibitor on the presence of SAM [167]. Later, an improved and highly specific compound, GSK3326595 (EPZ015938), was developed which is undergoing three clinical trials[168].

2.4.2 SAM Competitive Inhibitors

This class of inhibitors also called SAM mimetics antagonizes PRMT5 enzymatic functions by competing with SAM for binding to PRMT5. Compound 1 also called LLY-283, is the first SAM competitive inhibitor identified to antagonize PRMT5 function and has both *in vivo* and *in vitro* anti-PRMT5 effects with IC50 of around 20 nM. LLY-283 suppressed tumor growth in mouse xenograft model of melanoma[169]. In another study, LLY-283 caused aberrant splicing in GBM stem cells and crossed the blood-brain barrier in mice with orthotopic xenografts of GBM stem cells, ultimately improving the longevity of these mice by thwarting tumor development[170]. A dual PRMT5-PRMT7 inhibitor, DS-437, was characterized as a SAM-competitive inhibitor, though it was more specific for PRMT7. Nevertheless, it achieved significant anti-PRMT5 effects *in vitro*, with an IC50 value of about 6 μM. This inhibitor was proposed as template for future PRMT5-PRMT7 inhibitors design[171]. The screening of the ChemBridge CNS-Set library, consisting of 10,000 small molecule compounds (CMPs), led to the discovery of CMP5 (compound 5) as a highly selective small molecule inhibitor of PRMT5. This inhibitor was tested against EB-virus induced B-cell lymphoma and demonstrated relative specificity against PRMT5 compared to PRMT1 or PRMT7. Escalating concentrations of CMP5 was cytotoxic to lymphoma cells, but relatively tolerable by normal lymphocytes[91]. Similarly, PJ-68 was identified through screening with IC50 of 517nM against PRMT5 activity in CML cell lines. PJ-68 extended life of mice with leukemic stem cells compared to vehicle treated cells[92]. Another SAM mimetic inhibitor called compound 9 was identified to interact covalently with cysteine

449 of PRMT5 to suppress its enzymatic function[172]. These inhibitors have paved way for several SAM competitive inhibitors, of which some are in clinical trials.

2.4.3 PRMT5:MTA Inhibitors

PRMT5 is also endogenously inhibited by MTA, a byproduct of the polyamine biosynthesis that is metabolized by MTAP. MTAP is commonly deleted with p16/CDKN2A in most cancer cells. By knocking down several genes in *MTAP* wild type and *MTAP* null cancer cells, PRMT5 was identified as one of the synthetic lethal in the context of MTAP deficiency. *MTAP* deletion leads to the accumulation of MTA, which in turn inhibits PRMT5 enzymatic activity. This inhibitory effect is further diminished by either knocking down or inhibiting PRMT5. Interestingly, inhibiting PRMT5 enzymatic activity using EPZ015666 could not recapitulate the PRMT5 knockdown in *MTAP* knockout cancer cells[173]. To recapitulate the knockout impact of PRMT5 on tumor growth, the first PRMT5 degrader was identified and characterized as Compound 15 (MS4322). This compound was developed by linking the PRMT5 inhibitor EPZ015666 to the von Hippel-Lindau (VHL) E3 ligase ligand, promoting the degradation of PRMT5. Indeed, examining whole cell lysates from cells treated with this PRMT5 degrader showed reduction of PRMT5 and MEP50, attesting to its specificity. Remarkably, this inhibitor exhibited dramatic antitumor efficacy against different cancer types[174].

As promising as these PRMT5 degraders may be, inevitable toxicities persist, as the degraders cannot differentiate between normal PRMT5 and cancer-associated PRMT5. Hence, the newer generation of PRMT5 inhibitors is designed to bind and stabilize PRMT5

in complex with MTA in *MTAP*-deleted tumors, thereby enhancing the inhibition of PRMT5 enzymatic functions. One example is MRTX1719 which has exhibited remarkable antitumor response preclinically across different cancer types[184]. Importantly, patients with MTAP-null solid tumors, when administered oral doses of MRTX1719, have shown no adverse effects so far, with partial responses recorded across different tumors. Thus, PRMT5:MTA based inhibitors appear to be the future of PRMT5 inhibitors[175,176].

2.4.4 RMT5 Inhibitors in Clinical Trials

There have been about eight PRMT5 inhibitors in clinical trials at various phases so far (**Table 2.3**).

AMG is an oral MTA-co-operative PRMT5 inhibitor that preferentially targets the MTA-bound state of PRMT5 in *MTAP* null tumors. It is currently in clinical trials, either in solitude or in combination with the anti-cell cycle agent docetaxel, for the treatment of *MTAP* null solid tumors such as non-small cell lung cancer (NSCLC), pancreatic adenocarcinoma, biliary tract cancer, and head and neck squamous cell carcinoma (HNSCC)[177].

GSK3326595 is an optimized form of EPZ01566 that is extremely specific for PRMT5 compared to other methyltransferase inhibitors[168]. This inhibitor is undergoing three different clinical trials. Trial 1 (NCT02783300) is a completed phase 1 trial involving patients with advanced or recurrent solid tumors and NHL. Published results indicate that most enrolled patients had adenoid cystic carcinoma, breast cancer, and colorectal cancer. Participants were administered inhibitor concentrations ranging from 2.5 mg to 600 mg

once daily (QD), and from 50 mg to 200 mg twice daily. The recommended phase 2 dose (RP2D) was determined to be 400 mg QD. Adverse events were observed in more than 90% of patients, including fatigue, anemia, thrombocytopenia, neutropenia, alopecia, and dysgeusia. The efficacy of the inhibitor was demonstrated by a reduction in SDMA levels in plasma and tumor samples from patients. Overall clinical activity was recorded in most of the cancers. Phase II is ongoing, but detailed information is not widely available[178]. Trial 2 (NCT03614728) aims to assess the safety and clinical activity of GSK3326595 in patients with relapsed and refractory Myelodysplastic Syndromes, chronic myelomonocytic leukemia (CMML), and AML. The study is divided into three parts: the first part evaluates the positive response to the inhibitor as monotherapy, the second part assesses the safety and clinical activity of GSK3326595 in combination with 5-Azacitidine. The third part, intended to test GSK3326595 as monotherapy in patients with relapsed or refractory AML harboring mutations in the mRNA splicing machinery, did not commence, likely due to the prevalent toxicities associated with the clinical program for GSK3326595[179]. The third trial (NCT04676516) was a phase 2 random trial evaluating GSK3326595 as monotherapy in early-stage breast cancer. This study is completed but no results is posted[5].

JNJ-64619178 is an irreversible PRMT5 oral inhibitor that binds SAM and the substrate binding pocket of PRMT5 (dual SAM and substrate competitive inhibitor) to interfere with PRMT5 enzymatic activities. This phase I trial was conducted to determine the maximum tolerated dose of JNJ-64619178 in patients with relapsed or refractory B cell NHL or advanced solid tumors and establish RP2D. Among the 54 enrolled patients, common cancers included adenoid cystic carcinoma, prostate cancer, and uveal

carcinoma. Patients were administered intermittent doses ranging from 0.5 mg to 4 mg or 1 mg to 2 mg daily, with the 1.5 mg intermittent and 1 mg daily dose favored for RP2D. Common treatment-related toxicities included anemia, nausea, thrombocytopenia, neutropenia, fatigue, asthenia, and diarrhea. JNJ-64619178 effectively targeted PRMT5, as evidenced by reduced circulating SDMA[180].

PF-06939999 is another oral PRMT5 inhibitor whose complete mechanism has not been fully elucidated. A Phase I dose escalation and safety trial has completed in patients with the following tumor types: metastatic endometrial cancer, HNSCC, NSCLC, urothelial cancer, cervical cancer, or esophageal cancer. Anemia, fatigue, neutropenia, thrombocytopenia, dysgeusia were the common adverse drug events documented. Like other PRMT5 inhibitors in clinical trials, target engagement was achieved when plasma SDMA was quantified. Importantly, no treatment related death has been recorded with 6mg daily established as RP2D[181].

In the trial involving PRT543, an oral substrate competitive inhibitor, the objective was to determine a safe dose for patients with advanced solid and hematological tumors who did not respond to existing treatment options. While the study has been completed, results are currently pending and have not been posted[182] .

PRT811, developed by Prelude Therapeutics, has demonstrated the ability to penetrate the blood-brain barrier and suppress human glioblastoma multiforme (GBM) orthotopic xenografts in mice[183]. This ongoing phase I dose-escalation trial aims to treat patients with advanced tumors and high-grade gliomas who have exhausted available treatment options. The doses administered ranged from 15 to 600 mg daily or 300 mg

twice daily. As of 2021, an equal number of patients with advanced solid tumors and glioblastoma have enrolled in the trial. Reduced serum SDMA levels and increased intron retention in certain genes indicate the efficacy of PRT811 in reducing PRMT5 enzymatic function. Notably, partial responses have been observed in some GBM patients, suggesting the inhibitor's ability to cross the blood-brain barrier. Some patients with solid tumors showed stable disease. Adverse drug events include anemia, vomiting, diarrhea, constipation, pruritis, nausea, and lymphopenia, with no treatment-related deaths reported[183,184].

In summary, while PRMT5 inhibitors have demonstrated significant anti-PRMT5 effects, there have been varied reports on their antitumor efficacy. Furthermore, the numerous toxicities associated with these inhibitors have posed challenges in advancing some trials, raising additional concerns about their safety.

2.4.5 Combination Therapy Involving PRMT5 Inhibitors

Due to the observed toxicity and increased resistance in various monotherapy approaches in cancer treatment, seminal studies are focused on identifying pathways or substrates that can be targeted in combination therapy to enhance the efficacy of monotherapy. In the case of PRMT5, the toxicities documented in clinical trials highlight the need for ways to mitigate these side effects. Preclinical studies using *in vitro* and mouse models have demonstrated that targeting pathways regulated by PRMT5, in conjunction with PRMT5 inhibitors, enhances the antitumor efficacy of PRMT5 inhibitors. PRT382 demonstrated an antitumor effect against the MCL-xenograft model. Treatment of MCL cells with PRT382

resulted in a decreased interaction between AKT and FOXO1, leading to increased translocation of FOXO1 into the nucleus. This promoted the transcription of pro-apoptotic genes such as BAX. The combination of Venetoclax, an FDA-approved drug for treating CML that inhibits the anti-BAX protein BCL-2, with PRT382 showed significant antitumor synergy in MCL PDX models[185]. Furthermore, the PRMT5 inhibitor AMI-1 demonstrated synergy with the DNA damage-inducing agent Cisplatin in lung cancer cells, resulting in a significant reduction in cell viability compared to treatment with either reagent alone. The combination treatment arrested cells at the G1 phase of the cell cycle and increased cell death. It was suggested that the combination of Cisplatin and the PRMT5 inhibitor led to increased shuttling of PRMT5 from the nucleus to the cytoplasm, resulting in reduced H4R3me2s[186]. In GBM mTOR inhibitors were found to stimulate PRMT5-mediated symmetric dimethylation of hnRNPA1, leading to the induction of internal ribosome entry site (IRES)-dependent translation. Consequently, PRMT5 was identified as a key factor contributing to resistance to mTOR inhibitors in GBM. To address this resistance, a combination treatment strategy was employed, targeting PRMT5 with the EPZ01566 inhibitor in conjunction with the mTOR inhibitor. Notably, this combination significantly reduced the development of GBM xenograft tumors and increased overall survival in mice, highlighting the potential efficacy of dual targeting to overcome resistance to mTOR inhibitors[187]. In another study, considering the regulatory role of PRMT5 in the proliferation and function of Treg cells in peripheral lymph nodes, it was reported that the combination of PRMT5 inhibitors DS-437 and EPZ015666 enhanced the cytotoxic effect of

Table 2.3. PRMT5 inhibitors in clinical trials

Inhibitor	Company/Sponsor	Cancer type	Phase/status	Adverse effects reported	Clinicals trial identifier
AMG 193	Amgen	*MTAP* null solid tumors	Phase 1 (Active)	Not posted	NCT05094336
GSK3326595	GlaxoSmithKline	Relapsed and refractory MDS, chronic myelomonocytic leukemia (CMML), and AML	Phase 1 (Terminated)	Not posted	NCT03614728
GSK3326595	GlaxoSmithKline	Solid tumors and non-Hodgkin's lymphoma (NHL)	Phase 1&2 (completed)	Anemia, thrombocytopenia, neutropenia, alopecia, dysgeusia.	NCT02783300
GSK3326595	GlaxoSmithKline	Breast Cancer	Completed	Not reported	NCT04676516
JNJ-64619178	Janssen Research & Development	Advance solid tumors	Phase 1(Active)	Anemia, nausea, thrombocytopenia, neutropenia, fatigue, asthenia, and diarrhea	NCT03573310
PF-06939999	Pfizer	Solid tumors	Phase 1(terminated)	Anemia, fatigue, neutropenia, thrombocytopenia, dysgeusia	NCT03854227
PRT543	Prelude Therapeutics	Solid and hematological malignancies	Phase 1(completed)	Not posted	NCT03886831
PRT811	Prelude Therapeutics	Glioblastoma	Phase 1(completed)	Anemia, vomiting, diarrhea, constipation, pruritis, nausea and lymphopenia	NCT04089449

the p185erbB2/neu ectodomain-targeted monoclonal antibody. This effect was observed even in a mouse breast cancer model that was resistant to anti-p185erbB2 antibody[131].

In MLLr, the histone lysine methyltransferase DOT1L and PRMT5 are implicated in promoting tumorigenesis by increasing the expression of genes involved in stemness and suppressing apoptosis, respectively. Thus, PRMT5 inhibitors sensitized MLLr cells to DOT1L inhibitors, with combination treatment dramatically reducing proliferation and inducing cell death compared to single treatment. It is noteworthy that the combination of PRMT5 and DOT1L inhibitors further sensitized MLLr cells to Cytarabine, which is a chemotherapy medication for lymphomas and leukemia[188]. PRMT5 also antagonize antitumor immunity by promoting the expression of PD-L1 on tumor cells and decreasing the infiltration of antitumor immune cells into the tumor microenvironment. PRMT5 responded to INF-γ to induce expression of STAT1 by dimethylating H3R2 on its promoters. It is believed the JAK2/STAT1 pathway drives PD-L1 expression[189]. In agreement, combination of PRMT5 inhibitors and anti-PD1 antibody synergistically suppressed mice model of melanoma[190]. Similarly, because PRMT5 is crucial for AKT activation, combining AKT and PRMT5 inhibitors was extremely cytotoxic across different breast cancer cells[9]. Likewise, inhibiting tyrosine kinase using imatinib synergized with PRMT5 inhibitor PJ-68[92].

Against this backdrop, some of the PRMT5 inhibitors in clinical trials will test how combinations with other inhibitors can improve their efficacy. For example, the second part of NCT03614728 evaluated the safety and clinical activity of GSK3326595 in combination with 5-Azacitidine, which stimulates the expression of tumor suppressors. Additionally, two trials, NCT05094336 and NCT03854227, will test the anti-cell cycle

inhibitor docetaxel in combination with PF-06939999 and AMG 193 PRMT5 inhibitors in solid tumors.

Overall, due to the high toxicities associated with PRMT5 inhibitors, pairing them with inhibitors of pathways regulated by PRMT5, such as splicing, immune response, or cell signaling, appears to be a promising avenue for improving the efficacy of PRMT5 inhibitors in treating cancers and potentially minimizing toxicities.

Chapter 3: Autophagy in Breast Cancer

3.1 Autophagy Process

Autophagy is an evolutionarily conserved eukaryotic process of maintaining cellular homeostasis under normal and hostile conditions. It involves packing target substrates which could be proteins, lipids, nucleic acids, pathogens, organelles, or whole cells into double-membraned vesicles called autophagosomes and fusing them with lysosomes (**Figure 3.1**)[191]. Lysosomal degradation of autophagy substrates serves as a mechanism to recycle nutrients under starvation conditions and protect cells from the toxic effects of misfolded proteins or damaged organelles. Consequently, autophagy impairment has been implicated in neurogenetic disease, cancer and other disease conditions[192]. There are three forms of autophagy in mammalian cells: macroautophagy, microautophagy and chaperone-mediated autophagy[193,194]. While macroautophagy (hereafter referred to as autophagy) involves de novo synthesis of double-membraned vesicle that engulf cytoplasmic components, microautophagy involves the lysosomal membrane sequestering cytoplasmic component. Chaperone-mediated autophagy (CMA) on the other hand employs chaperones that recognize and bind unique pentapeptide motif (KFERQ) exposed on target proteins thereby delivering them to the lysosome[195,196]. The autophagy pathway has been divided into five steps; namely: initiation, membrane nucleation, membrane elongation, vesicle fusion and cargo degradation. Each step is coordinated by variety of proteins called autophagy related (ATG) proteins[197]. Several intracellular and extracellular signals such as scarcity of nutrients, DNA damage and growth factors deficiency evoke autophagy[198-200].

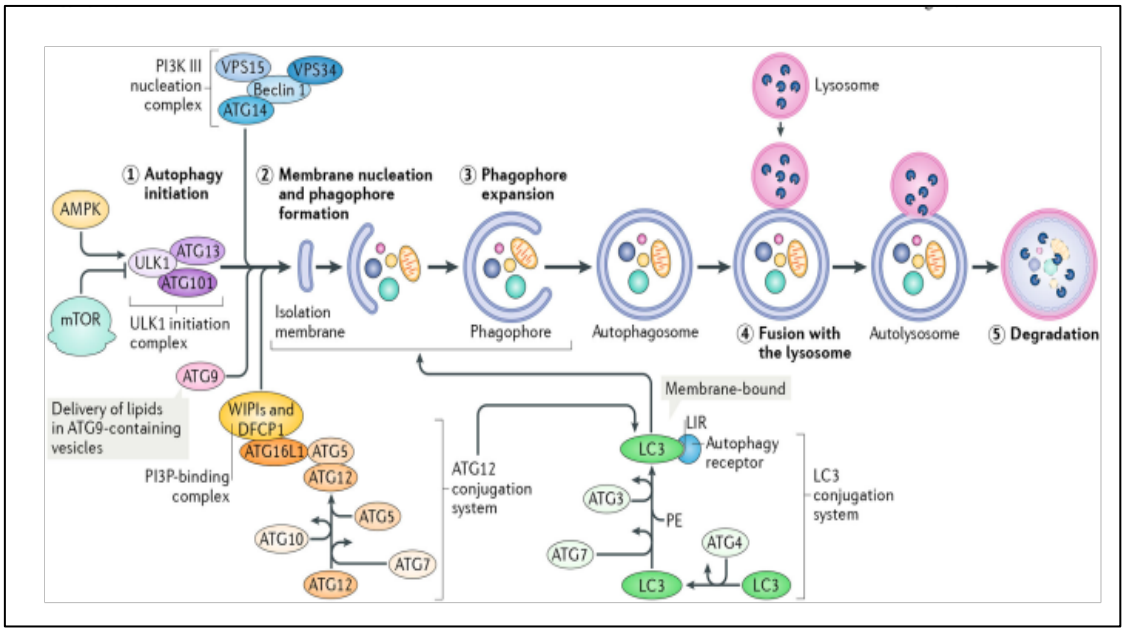


Figure 3.1. Stages of the autophagy process (adopted from[191])

The initiation stage occurs largely at endoplasmic reticulum (ER)[201] though mitochondrion, Golgi apparatus and plasma membrane have been documented as potential sites[202-204]. Initiation of autophagy requires the activation and assemblage of ULK1/ULK2 complex which is a composite of ULK1/ULK2, ATG13, FIP200 and ATG101. Activated ULK1 complex migrates to specific sites marked by ATG9[205] to nucleate the autophagosome. The class III PI3K complex, consisting of Beclin 1, PIK3C3/VPS34, PIK3R4/VPS15, ATG14, and NRBF2[206,207], is subsequently activated by ULK1. This activation leads to the formation of PtdIns3P-rich structures known as omegasomes, which serve as platforms for recruiting other autophagic proteins required for phagophore or isolation membrane formation[208]. The activity of the Beclin 1/PIK3C3/VPS34 complex is regulated by their interacting proteins: UVRAG and SH3GLB1 promote its catalytic activity while partners like RUBCN and BCL2 inhibit it.

PtdIns3P and its binding proteins like WIPI localize to the phagophore to facilitate the elongation phase. This phase involves two ubiquitin-like conjugating systems: ATG12-ATG5 and ATG8/LC3B. ATG12 is activated by ATG7, acting like an E1-like activating enzyme, and then transferred to ATG10, which functions as an E2 conjugating enzyme. Eventually, ATG12 is bonded to an internal lysine of ATG5 to form the ATG12-ATG5 pair. This pair, along with ATG16L, forms a complex that attaches to the phagophore[206,209]. The ATG12-ATG5-ATG16L complex recruits LC3 to the phagophore[210-212] where LC3 is subsequently cleaved by a cysteine protease, ATG4B, exposing a glycine residue that permit ATG7 to activate it. Upon activation, LC3 is transferred to ATG3 (E2-like conjugating enzyme) where it is finally conjugated to phosphatidyl ethanolamine

mediated by the ATG12-ATG5 pair[212,213] to form LC3-II and incorporated into the growing autophagosome[214]. LC3-II also serves as a receptor for proteins that possess an LC3-interacting region, allowing them to be delivered to the autophagosome. Its accumulation is an accepted marker of autophagy induction.

Matured autophagosomes either directly fuse with lysosomes or fuse with late endosomes, which then fuse with lysosomes to degrade their content. Fusion of lysosomes and autophagosomes is mediated by small GTPase and SNARE proteins that facilitate the migration of autophagosomes and lysosomes toward each other[215,216] . Upon coming together, the outer membrane of the autophagosome fuses with the lysosomal membrane, exposing its content to the lumen of the lysosome for degradation [217]. Receptors like P62/SQSTM1, NBR1, TAXIBP1, NDP52 and OPTN recruit ubiquitinated substrates to the lysosome via their LC3 interacting region[218,219]. Some chemical agents like bafilomycin A1 block this degradative stage by decreasing the acidity of the lysosome while chloroquine (CQ) impairs autophagosome-lysosome fusion[220].

3.2 Breast Cancer Subtypes

Breast cancer is the most frequent cancer among women, with approximately 300,000 estimated new cases to be diagnosed in the US for the year 2023[221]. Furthermore, it is forecast to be the second leading cause of cancer death, warranting improved preventive and curative strategies to address it. Breast cancer is categorized into different subtypes, including luminal tumors (luminal A and B), which are positive for estrogen (ER+) and/or progesterone receptors (PR+); HER2, characterized by the overexpression of the ERBB2

oncogene; and triple-negative tumors (TNBC), which lack hormone receptors and HER2 enrichment.

Hormone receptor-positive breast cancers, specifically the luminal A and B subtypes, make up approximately 70% of all diagnosed breast cancers and are highly responsive to endocrine therapy. Luminal B tumors, in comparison to luminal A, exhibit characteristics such as low expression of estrogen receptor (ER) genes, low or no progesterone receptor (PR) expression, high tumor grade, elevated expression of proliferation genes, and activation of growth factor receptor signaling pathways such as IGF-1R and PI3K/AKT/mTOR pathways[222].

The HER2-enriched subtype of breast cancer expresses the HER2 transmembrane receptor tyrosine kinase from the EGFR family. This subtype activates signaling pathways, including MAPK, JAK/STAT, RAS/MEK/ERK, and PI3K/AKT/mTOR, which are implicated in cellular proliferation and differentiation in cancers[223]. HER2-enriched tumors constitute about 15% of all breast cancers and are responsive to targeted anti-HER2 therapies such as Trastuzumab, Trastuzumab emtansine (T-DM1), Lapatinib, Pertuzumab, and Afatinib[224].

TNBC tumors account for about 15% of all breast cancer with cytotoxic chemotherapy being the cardinal treatment option[225]. They are characterized by aggressive phenotype ,early relapse and poor prognosis[226]. Most TNBC tumors harbors high frequency of TP53 mutations or loss of the p53 pathway activity, loss of *RB1* and *BRCA1*, leading to impaired DNA damage repairs[227], as well as aberrant activation of signaling pathways including the PI(3)K/AKT, MYC and FOX1M, all of which contribute to its highly aggressive and metastatic nature[228].

3.3 Role of Autophagy in Breast Cancer

Autophagy is generally believed to have a binary role in tumorigenesis: being a tumor suppressor or tumor promoter. The initial indication of autophagy as a tumor suppressor in breast cancer stemmed from findings that monoallelic deletion of *BECN1* is observed in approximately 40-75% of breast and ovarian cancers. Additionally, the evidence was supported by the observation that overexpression of Beclin1 led to a reduction in *in vitro* clonogenicity and tumor formation in nude mice[229,230]. In addition, analysis of the TCGA data base has showed low *BECN1* mRNA is associated with HER2-enriched and basal like breast cancer tumors[231]. Indeed, HER2 was shown to interact with and inhibit Beclin1 autophagic activity in HER2+ breast cancer cells. Mice with homozygous knock-in mutation of Beclin1 that abrogates its interaction with HER2 had longer life span and reduced tumor burden[232]. Additionally, mice with heterozygous deletion of Becn1 exhibited twice the incidence of sporadic malignancies, including mammary tumors, compared to their wild-type counterparts[233,234]. Thus, much of the tumor-suppressive role of autophagy in breast cancer is associated with the loss of Beclin 1. However, the assertion that Beclin 1 serves as a suppressor in breast cancer settings is debatable due to the rarity of biallelic loss of Beclin 1 and co-deletion of Beclin 1 and BRCA1, based on their proximity on chromosome.

The protumorigenic role of autophagy in breast cancer development has also been documented. Specifically, FIP200 has been identified as a promoter of breast tumorigenesis by inhibiting antitumor immune surveillance. In this study, the knockout of FIP200 resulted in reduced colony formation, proliferation, and metastasis of breast

tumors in the MMTV-PyMT mouse model of breast cancer. This model induces mammary tumorigenesis through PyMT-guided activation of RAS, Src, and PI3K signaling pathways[235]. In a similar study using conditional deletion of *palb2* in the mammary gland that yielded various mammary tumors, heterozygous loss of *Becn1* inhibited the early onset and the number of breast tumors. It was believed that loss of *palb2* evoked DNA damage stress that required cytoprotection from autophagy, which when blocked, increased cell death to reduce tumor growth[236]. In a context-dependent manner, TNBC that possess oncogenic K-RAS mutation are 'addicted' to autophagy. Under such addictions, the uncontrolled proliferation, and its associated energy demand as well as accompanying stress make autophagy critical for survival. Accordingly, knockdown of *ATG7* reduced the colony formation and metastatic potential of MDA-MB-231 cells[237].

Several studies have implicated autophagy or ATG genes as contributory factors to TNBC phenotype. TNBC have been reported to have high basal autophagy evidenced by increased expression of LC3B compared to normal and luminal breast cancer cells. This high basal autophagy could be due harsh environment like hypoxia that are associated with TNBC tumors[238]. Similarly, by comparing different subtypes of breast cancer tumors, TNBC emerged as having the highest expression of LC3A, LC3B, Beclin 1 and ATG9[239,240]. This high LC3B and other ATG expression was believed to be a contributory factor to the aggressiveness, metastasis, and worse prognosis of TNBC[241-243]. In fact, knockdown of LC3B and Beclin 1 reduced cell viability, migration, clonogenicity of TNBC cells[243] just as knockdown of ATG7, ATG5 and Beclin 1 as well as CQ treatment severely impaired clonogenicity and growth of TNBCs compared to nontumorigenic and luminal breast

cancers, indicating TNBCs were more dependent on autophagy for survival and growth[244]. This evidence highlights the instrumental role of autophagy to TNBC survival and proliferation and how inhibiting autophagy genetically or chemically impede TNBC tumorigenesis.

3.4 ULK1 in Autophagy

ULK1 is a conserved serine-threonine kinase, analogous to Atg1 in yeast that orchestrates the molecular arrangement and assemblage of ATG proteins in forming the phagophore. It serves as nexus in receiving and relaying upstream signals from mTORC1 and AMPK which reflects the nutrient and energy status of the cells, respectively. There are five mammalian ULK homologs (ULK1, ULK2, ULK3, ULK4, and STK36). However, only ULK1 and ULK2 are putative autophagy proteins. Interesting, only ULK1 and ULK2 share greater sequence similarities and have some redundancy in autophagy[245,246]. Yet, homolog specific functions have been reported. For example, ULK1 was super critical for autophagy-mediated clearance of mitochondrion and ribosomes in erythrocytes[247]. ULK1 has a kinase domain (KD) responsible for the enzymatic function and C-terminal interacting domain (CTD) sandwiching a serine-proline or intrinsically disordered region (IDR) which is prone to several PTMs (**Figure 3.2**). Indeed, more than 40 phosphorylation sites have been identified in ULK1, with many of the residues residing in the IDR region[248]. The CTD domain possess a microtubule-interacting transport (MIT) domain required for binding membrane, ATG13 and FIP200[249,250]. It is believed that ULK1 exists as a constitutive complex with ATG13 and FIP200 and that ATG13 and FIP200 are required for

ULK1 stability and enzymatic function[250-252], though contrasting data indicates the complex is indispensable for autophagy[253].

3.5 Regulation of ULK1 Activities

ULK1 activities are regulated transcriptionally to affect its protein expression or through PTMs to alter is stability, interactions, or enzymatic function. Several PTMs, notably phosphorylation (**Table 3**), fine-tune the autophagy process. In nutrient replete environment, ULK1 is inactivated by mTORC1-mediated phosphorylation and becomes dephosphorylated during starvation to become active[245]. Under energy-deficient conditions, AMPK activates ULK1 by directly phosphorylating and indirectly antagonizing mTORC1-mediated inhibition of ULK1[254-256]. Activated ULK1 autophosphorylates to enhance its kinase activity[257] and activate ULK1 complex and Beclin 1[258,259] to initiate autophagosome formation and nucleation, respectively (**Figure 3.3**). ULK1 is also acetylated. Acetylation of ULK1 is germane for serum-starvation induced autophagy but not glucose-starvation induced autophagy[260], indicating how different upstream signals modulate ULK1 modifications to induce autophagy under specific stress conditions.

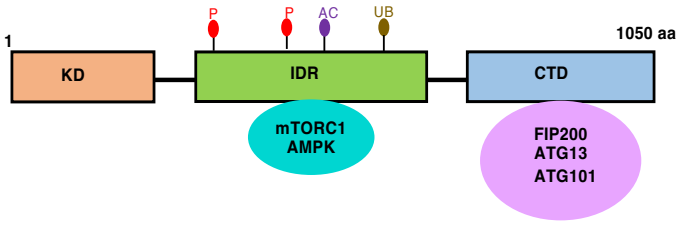


Figure 3.2. Schematic of human ULK1 domains. The IDR is subject to PTMs like phosphorylation (P), acetylation (AC), and ubiquitylation (UB) and serves the docking site for upstream regulators like mTORC1 and AMPK. The CTD is where members of the ULK1 complex (FIP200, ATG13, and ATG101) bind.

Some evidence shows that ULK1 autophagic activities is not constitute in autophagy. At the transcriptional level, ATF4 directly upregulate ULK1 mRNA and protein expression under hypoxia and ER stress[261,262].During amino acid or serum starvation induced autophagy, ULK1 protein expression vacillates as it undergoes lysosomal and proteasomal degradation mainly through ubiquitylation as means of modulating its kinase and autophagic functions. Ambra 1-TRAF6 E3 ligase-mediated ULK1 K63 ubiquitylation stabilizes ULK1 and promotes ULK1 autophosphorylation required for autophagy initiation upon amino acid starvation[263]. It is believed that the TRAF6-mediated ubiquitylation of ULK1 stabilizes ULK1 for the first 30 minutes of starvation-induced autophagy after which ULK1 is released to allow NEDD4L-mediated K48 ubiquitylation to target ULK1 for proteasomal degradation[264], as a means of dampening the autophagy process. ULK1 not only initiates autophagy but sometimes functions in autophagosome and lysosome fusion. It has been reported that phosphorylation of ULK1 by PKCα impairs autolysosome formation to block degradation state of autophagy. Additionally, phosphorylated ULK1 is degraded by chaperone-mediated autophagy, serving as a mechanism of regulating macroautophagy[265]. This dynamic functions of ULK1 highlight the convoluted role of ULK1 in autophagy. At the later stage of autophagy ULK1 is degraded via Cul3-KLHL20-mediated K48 ubiquitylation to terminate the process[266].

Thus, it appears that ULK1 is very critical throughout the whole autophagy process. However, instances of ULK1-independent autophagy have been recorded. For example, neurons isolated from mice deficient in ULK1/2 did not display abnormal accumulation of mitochondrion or ubiquitinated inclusions, suggesting intact

Table 3. Key modifications of ULK1

PTM	Effector	Consequence	References
Phosphorylation: T180,(S1042, T1046) Phosphorylation: S637, S757	ULK1 mTOR	Activates ULK1 Kinase activity and promotes autophagy termination respectively. Inhibit ULK1 kinase activity.	254,256,257,266
Phosphorylation: S317, S467, S55, S574, S673, S659, S777 S423	AMPK PKCα	Promote ULK1 activation. Prevents autolysosome formation.	254,256 248, 259,265
Acetylation: K162, K606	TIP60	Activates ULK1 activity under serum-starvation induced autophagy.	260
Poly-ubiquitylation: K48 K48 K63	CUL3-KLHL20 NEDD4L TRAF6	Promotes ULK1 degradation to terminate autophagy. Promotes ULK1 degradation to dampen the autophagy process. Stabilizes ULK1 protein during initiation of autophagy process.	266 264 263

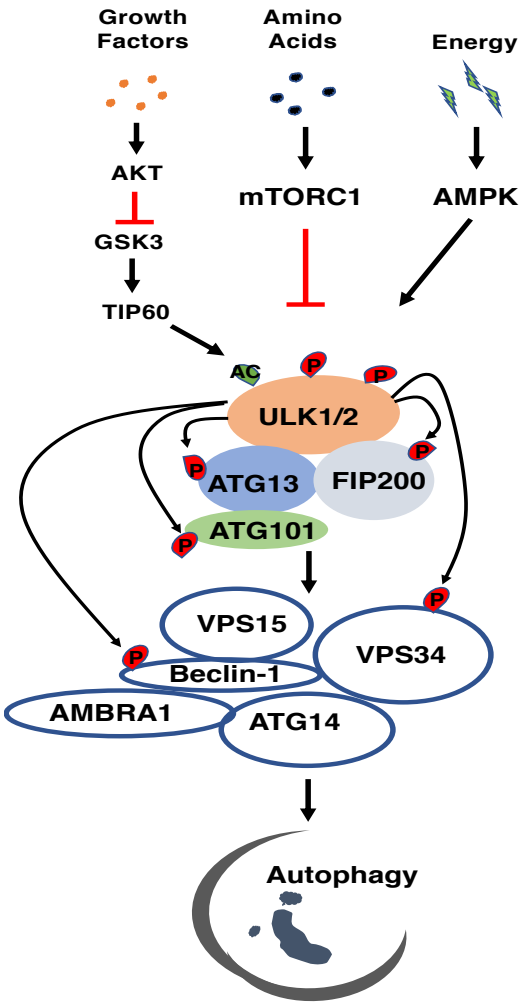


Figure 3.3. Schematic of how ULK1 response to upstream stress signals. Stress cues emanating from amino acid, glucose or growth factor scarcity activate ULK1 kinase, leading to phosphorylation of several downstream substrates to promote autophagy.

suggesting intact autophagy[267]. Again, mouse embryonic fibroblast depleted of ULK1/2 were capable of inducing autophagy under ammonia stress[268].

3.6 ULK1 In Cancer

ULK1 mutations have not been interrogated extensively; however, ULK1 overexpression has been reported in several cancers, usually correlating with a negative prognosis. In ovarian cancer, ULK1 overexpression was observed, and knockdown of ULK1 inhibited proliferation following reduced autophagy in ovarian spheroids, but not in normal monolayer cells[269]. Similarly, high ULK1 expression negatively correlated with survival and prognosis of several cancer types including breast, esophageal squamous carcinoma, gastric, colorectal and NSCLC. Notably, knockdown of ULK1 reduced autophagy and caused apoptosis in these cancer types[262,270-273]**.** Despite these reports, ULK1 autophagy-independent functions have been identified in cancer development. For example, nuclear ULK1 induce cell death independent of autophagy by activating PARP1 upon oxidative stress[274]. Additionally, ULK1 impedes metastasis by phosphorylating and inhibiting Exo70 oligomerization required during cell migration[275] .

3.7 ULK1 Inhibitors

The cardinal role of ULK1, particularly in starvation and stress conditions, coupled with its druggable nature due to successful usage of kinase inhibitors, has made it important target in blocking autophagy. Over the years, several small molecule inhibitors that target ULK1 ATP binding site have been developed. Lazarus et al. screened about 750 compounds

against ULK1 kinase activity using a standard ^{32}P-ATP radioactive assay with MBP as the substrate. This study discovered series of compounds that stabilized the enzyme and allowed for crystallization of the kinase domain[276]. However, this compound was not highly selective for ULK1, making it unsuitable for studying autophagy in cells. Later that year, the same group modified the PDK1 inhibitor BX-7952 to generate two compounds that inhibit ULK1 kinase activity with high selectivity and suitability for cellular studies[277]. The crystallized ULK1-kinase structure established from their previous study[276] was used in *silico* high throughput screen of about 600K ligands to identify SR-17398 with IC50 of about 20 μM against ULK1 activity[278]. Later, more highly specific, and selective ULK1 inhibitors with IC50 in nanomolar range have been reported. SBI-0206965 was identified through target-based reverse pharmacology approach where a library of pyrimidine analogs was screened to identify ULK1 kinase inhibitors. This inhibitor effectively suppressed ULK1 kinase and autophagic activity under starvation conditions with IC50 of 108nM and 711nM for ULK1 and ULK2 respectively. Importantly, SBI-0206965 enhanced the cytotoxicity of mTOR inhibitors in A549 lung cancer cells[279]. Martin et al. by screening existing pharmaceutical data also identified two closely related molecules, ULK-100 and ULK-101, as potent ULK1 inhibitors. This was evidenced by their ability to suppress ULK1 kinase functions, blocking ULK1-mediated Beclin 1 S15 phosphorylation, and inhibiting starvation-induced autophagy with an IC50 of about 2 nM, compared to 40 nM for SBI-0206965280.[280]. Importantly, these inhibitors suppressed K-RAS driven NSCLC that are autophagy addicted. Similarly, an *in vitro* screening discovered TBK1 inhibitor MRT67307 and MRT68921 as ULK1 and ULK2 inhibitors with the latter being the most potent with

IC50 of 2.9 nM and 1.1 nm for ULK1 and ULK2, respectively. These are ATP competitive inhibitors that block ULK1 kinase function both under normal and starvation conditions. Notably, MRT68921 was specific for ULK1 against a panel 80 human kinases[281].

In summary, over the years, numerous small molecule ULK1 inhibitors with varying cellular selectivity and specificity have been discovered. Among them, MRT6891 stands out as the most potent. However, it is noteworthy that none of these inhibitors have progressed to clinical trials.

Chapter 4: Materials and Methods

4.1 Cell Culture

All cells were obtained from American Type Culture Collection (ATCC). HEK293T, MDA-MB-231, MCF7, Hs 578T and their derived cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Genesee Scientific, 25–500). T-47D, MDA-MB-453, MDA-MB-468, BT-549, HCC70 and their derived cell lines were maintained in RPMI 1640 medium (Corning, 10-040-CV). 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin were supplemented in the medium. Cells were passaged every two days. During passage, cells were gently rinsed with phosphate-buffered saline (PBS) followed by addition of appropriate volume of trypsin/EDTA (0.5 ml for 60mm dish and 1 ml for 100mm dish) and incubated at 37 °C for 3-5 minutes. About 4-5 ml of RPMI or DMEM was added to neutralize the trypsin before splitting them into appropriate dishes. All cell lines used in this study were grown in cell culture vessels (flasks or dishes) in a humidified cell culture incubator with 5% CO2 at 37 °C.

4.2 Cell transfection, Virus Package, and Infection

For protein expression, transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, L3000001) according to the manufacturer's instructions. Briefly, for example when transfecting 2 ug plasmid DNA in 60mm dish, 6 µl of Lipofectamine 3000 was added to 375 µl of Opti-MEM medium (Invitrogen) and 4 µl of P3000 reagent was mixed with 2µg DNA in 375 µl of Opti-MEM medium and incubated for 5 minutes at room temperature, after which the Lipofectamine 3000 mixture was added to the P3000 -DNA

mixture, vortexed gently and allowed for 15 minute incubation at room temperature. The lipofectamine-DNA mixture was then added dropwise to HEK293T cells with 60% confluency. Medium was replaced 24 hours post transfection and cells were harvested the next day. For lentivirus production, target constructs containing sgRNA or cDNA were co-transfected with packaging plasmids (pMD2G and pSPAX2) into HEK293T cells with Polyethylenimine (PEI, Polysciences, 23966-1). Twenty-four hours post transfection, fresh medium was replaced. Virus containing supernatants were harvested at 48 h post transfection and filtered with 0.45 μm PES filter. Targeted cells were infected with virus and selected with hygromycin (200 μg/ml), puromycin (1–2 μg/ml) or blasticidin (10 μg/ml) for 4 days to eliminate the non-infected cells.

4.3 Plasmids

Flag-PRMT5, Flag-MEP50 were generated by cloning the corresponding cDNA into the pRK5-Flag vector while HA-PRMT5 and HA-ULK1 cDNA were cloned into the pRK5-HA vector. Myc-PRMT5, Myc-ULK1, Myc-Beclin 1, and Myc-Ambra1 were generated by cloning the corresponding cDNA into the pRK5-Myc vector. GST-Beclin 1 (1–86 aa) and GST-ULK1 (1–649 aa) were generated by inserting the cDNA into pGEX-6P-1 bacteria expression vector. Myc-ATG13 (#31965), Flag-FIP200 (#24300), GFP-LC3-RFP (#84573) were purchased from Addgene. Lentiviral HA-ULK1 and HA-PRMT5 were generated by cloning the corresponding cDNA into pTRIPZ-puro, pLenti-HA-hygro vector or pLJM1-HA-puro vector.

Various single guide RNAs (sgRNA) were designed at https://www.synthego.com and were cloned into lentiCRISPR v2 vector (Addgene, #52961). Sequence of sgRNAs and other PCR primers are provided in **Table 4.1**.

4.4 Antibodies

All primary antibodies were diluted with 5% non-fat milk in TBST buffer for Western blot (**Table 4.2**). Anti-ULK1 (8045), anti-Myc (2278), anti-cleaved Caspase 3 (9661), anti-AMPKα (5831), anti-Raptor (2280), anti-pS757-ULK1 (14202), anti-pS15-Beclin 1 (84966), anti-LC3B (3868), anti-ATG13 (13468), anti-PRMT1 (2449), anti-PRMT5 (79998), anti-S6K1 (9202), Rabbit anti-HA (3724), and anti-pT389-S6K (9234) were purchased from Cell Signaling Technology. Anti-Tubulin (66240-1-lg) and anti-Beclin 1 (11306-1-AP) were purchased from Proteintech. Rabbit anti-FLAG (F7425), mouse anti-FLAG (F3165), peroxidase-conjugated anti-mouse secondary antibody (A4416), and anti-rabbit secondary antibody (A4914) were purchased from Sigma. Monoclonal mouse anti-HA (901503) was purchased from BioLegend. Anti-PRMT7 (A12159) and anti-p62 (A11483) were purchased from ABclonal. Anti-pS318-ATG13 (600-401-C49) was purchased from ROCKLAND. Anti-MMA was a gift from Dr. Mark Bedford at MD Anderson Cancer Center.

4.5 Immunoprecipitation and Western Blot Analyses

Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and harvested with EBC buffer (50 mM Tris–HCl pH 7.5, 120 mM NaCl and 0.5% NP-40) when determining interactions or Triton buffer (40 mM HEPES pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM

EDTA and 1% Triton X-100) supplemented with protease inhibitor (Thermo Fisher, A32953) and phosphatase inhibitors (phosphatase inhibitor cocktail Set I and II, Calbiochem) and incubated at 4°C on a tube rotator for lysis. The cell lysates were centrifuged at 13,200 r.p.m. at 4 °C for 10 min. The protein concentration of lysates was determined using Nanodrop by Bio-Rad protein assay reagent. Equal amounts of whole cell lysates were resolved by SDS-PAGE and immunoblotted with indicated antibodies. For IP, 2000 – 5000 μg lysates were incubated with agarose conjugated antibodies for 3 – 5 hours at 4 °C. Immunoprecipitants were washed three times with NETN buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5% NP-40) or Triton buffer before being resolved by SDS-PAGE. Anti-HA agarose beads (A2095) and anti-FLAG agarose beads (A2220) were purchased from Sigma-Aldrich. Anti-Myc agarose beads (658502) were purchased from BioLegend.

4.6 Purification of Recombinant Protein from E. coli

Recombinant GST-ULK1 and GST-Beclin 1 truncated proteins were purified from the BL21(DE3) *Escherichia coli* transformed with corresponding constructs. Single colony was grown in 7 mL Luria–Bertani (LB) medium overnight at 37 °C. The culture was then inoculated into 400 mL LB medium until an optical density of 0.5–0.6. The protein expression was induced by 0.1 mM IPTG (isopropyl-β-D-thiogalactoside) at 25 °C for 16 hours. The bacteria cells were collected after centrifuging at 4000g for 10 minutes and re-suspended in GST buffer [25 mM Tris–HCl pH 8.0, 5 mM dithiothreitol (DTT), 150 mM NaCl] and sonicated. Sonicated bacteria pellets were centrifuged at 14,000 g for 35

minutes and the supernatant incubated with glutathione sepharose beads for 3 hours at 4 °C. The protein-bound glutathione beads were washed three times with GST buffer and recombinant GST proteins were eluted with elution buffer (10 mM L-Glutathione, 50 mM Tris–HCl pH 8.0 by gentle rotation at 4 °C for 10 minutes.

4.7 In vitro Methylation Assay

3 µg of recombinant GST-ULK1 truncated proteins were incubated with HA-PRMT5/MEP50 immunoprecipitated from 293T cells after transient transfection in a reaction mixture made up of methylation buffer (50 mM Tris–HCl pH 8.5, 20 mM KCl, 10 mM MgCl2, 1 mM β-mercaptoethanol, 100 mM sucrose) and 1 µL of adenosyl-L-methionine, S-[methyl-3H] (1 mCi/ml, Perkin Elmer) at 30 °C for 1 h. The reactions were stopped by 2 × SDS loading buffer and boiled at 100 °C for 10 minutes. The samples were resolved by SDS-PAGE and transferred to PVDF membrane, which was then sprayed with EN3HANCE (Perkin Elmer), kept at -80 °C for 2-3 weeks and exposed to X-ray film.

4.8 In Vitro Kinase Assay

3 µg of bacterially purified GST-Beclin 1 recombinant proteins were incubated with HA-ULK1 immunopurified from HEK293T cells in the kinase reaction buffer (25 mM HEPES pH 7.4, 50 mM NaCl, 5 mM MgCl2, 0.1 mM DTT, 0.5 mg/ml BSA) for 30 min at 30 °C. The reaction was stopped by adding 2 × SDS loading buffer. Samples were incubated at 100 °C for 5 min and resolved by SDS-PAGE. Phosphorylation of GST-Beclin 1 was detected by anti-pS15-Becllin 1 antibody.

4.9 Immunofluorescence Staining

Cells grown on glass coverslips to about 60% confluence were fixed with 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS, and then permeabilized with 0.05% Triton X-100 for 10 min at room temperature. Following three washes with PBS, cells were stained with DAPI, washed four times with PBS and mounted using vibrance antifade mounting medium (Vector Laboratories, H-1700). Images were taken by Leica SP8 Confocal microscope and puncta were counted manually.

4.10 Cell Viability Assay

Cells were seeded in 96-well plate at 500–1000 cells per well for 24 h and then treated with indicated doses of inhibitors for 4 days. Cell viability was determined using the Cell Titer-Glo cell viability assay kit according to the manufacturer's instructions (Promega, G7570). Briefly, 100µl of cell Titer-Glo cell viability reagent was added to each well, mixed by pipetting up and down and incubated at room temperature on a shaker for 15 minutes. Viability is measured by reading luminescence with PerkinElmer Victor3 plate reader.

4.11 Mass Spectrometric Analysis of ULK1-R532 Methylation

HEK293T cells were transfected with 6 µg HA-ULK1. Forty-eight hours post transfection, the cells were lysed in Triton buffer, followed by immunoprecipitation with ant-HA agarose beads. The immunoprecipitates were resolved by SDS-PAGE and visualized using GelCode blue staining reagent (Thermo Scientific, 24590). The protein band containing HA-ULK1 was excised and digested with trypsin. Peptides were analyzed on an EASY nLC 1200 in-

line with the Orbitrap Fusion Lumos Tribrid mass spectrometer. Peptides were pressure loaded at 800 bar and separated on a C18 reversed phase column (Acclaim PepMap RSLC, 75 µm × 50 cm (C18, 2 µm, 100 Å) (Thermo Fisher) using a gradient of 2–35% B in 180 min (Solvent A: 0.1% FA; Solvent B: 80% ACN/0.1% FA) at a flow rate of 300 nL/min at 45 °C. Mass spectra were acquired in data dependent mode with a high resolution (60,000) Fourier Transform mass spectrometry (FTMS) survey scan followed by MS/MS of the most intense precursors with a cycle time of 3 s. The automatic gain control target value was 4.0e5 for the survey MS1 scan. Precursors were isolated with a 1.6 m/z window with a maximum injection time of 50 ms. Tandem mass spectra were acquired using higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD) for each peptide precursor in an alternating fashion. The HCD collision energy was 35% and ETD was performed using the calibrated charge dependent ETD parameters. The fragment ions were detected in the Orbitrap at 15,000 resolution. Spectra were searched against a custom database containing human ULK1 and a database of common contaminants using MaxQuant and Proteome Discoverer. The false discovery rate, determined using a reversed database strategy, was set at 1% at the peptide and modification site levels. Fully tryptic peptides with a minimum of seven residues were required including cleavage between lysine and proline. Two missed cleavages were permitted. Sites of modification were manually verified.

4.12 Colony Formation Assay

Cells were seeded in 6-well plates at 300–500 cells per well for 24 h and then treated with indicated inhibitors for 8–10 days until visible colonies formation. Fresh medium with inhibitors was replaced every 3 days. Colonies were fixed with Fixer buffer(10% ethanol and 10% acetic acid) for 30 min and then stained with 0.4% crystal violent in 20% ethanol for 30 min, followed by washing with dH2O and counted.

4.13 PRMT5 and ULK1 Inhibitors

GSK3326595 (HY-101563), MRT68921 dihydrochloride (HY-100006A), and Chloroquine (HY-17589A) purchased from MedChemExpress. PRMT5 inhibitor was dissolved in DMSO while choroquine and MRT68921 were dissolved in sterilized water.

4.14 Site-Directed Mutagenesis

PRMT5-E444Q, ULK1-R532K and various ULK1 mutants were generated using the QuikChange XL site-directed mutagenesis kit. Briefly, PCR was performed with specific-tailored oligonucleotides to introduce the targeted mutation with the following PCR thermal cycling steps: 95 °C for 20 seconds to denature double-stranded DNA followed by 60°C for 30 seconds to anneal and 68 °C at 1min/kb to elongate. PCR products were digested with DpnI restricton enzyme for 2 hours at 37 °C. The final reaction was transformed into competent cells, plasmid was isolated from single colonies and sent for sequencing at Eurofins.

4.15 Statistical Analysis

As indicated in the figure legends, all quantitative data are presented as the mean ± SD of three biologically independent experiments or samples. Statistical analyses were performed using GraphPad Prism 9 and Excel. Statistical significance was determined by two-tailed Student's t test or two-way ANOVA. P value < 0.05 was considered significant.

Table 4.1. Sequence of sgRNAs and shRNA

PRMT5-sg1	GATGGAAGACAGGCATGCAG
PRMT5-sg2	ATGAACTCCCTCTTGAAACG
ULK1-sg1	GGAGAACTCGAACTTGCCCA
ULK1-sg2	AGCAGATCGCGGGCGCCATG
Beclin 1-sg1	GATCTTAGAGCAAATGAATG
Beclin 1-sg2	TGACAGTGAACAGTTACAGA
ATG13-sg1	GGTTACACATGAAGCAAAGA
ATG13-sg2	AGTCGGGAGGTCCATGTGTG
shULK1	GCCCTTTGCGTTATATTGTAT

Table 4.2. Antibodies

Antibody	Working Dilution(in 5% Milk)	Target size (KDa)
Rabbit anti- human ULK1	1:1000	150
Mouse anti-MYc	1:1000	N/A
Rabbit anti-human Cleaved Caspase3	1:1000	13,19
Rabbit anti- human AMPKα	1:1000	62
Rabbit anti- human Raptor	1:1000	150
Rabbit anti-mousepS757-ULK1	1:1000	150
Rabbit anti-human Beclin 11S15	1:5000	60
Rabbit anti-human LC3B	1:10,000	14,16
Rabbit anti-human ATG13	1:1000	72
Rabbit anti-human PRMT1	1:1000	41
Rabbit anti-human S6K1	1:1000	70, 85
Rabbit anti-human PRMT5	1:1000	73
Mouse anti-HA	1:5000	N/A
Rabbit anti-human pT389-S6K	1:1000	70, 85
Mouse anti-human Tubulin	1:10,000	56
Rabbit anti-human Beclin 1	1:5000	54
Rabbit anti-FLag	1:3000	N/A
Mouse anti-Flag	1:1000	N/A
HRP-linked goat anti-mouse IgG	1:3000	N/A
HRP-linked goat anti-rabbit IgG	1:3000	N/A
Rabbit anti-HA	1:1000	N/A
Rabbit anti-human PRM7	1:1000	78
Rabbit anti-human p62	1:10,000	62
Rabbit anti-human pS318-ATG13	1:1000	72

Chapter 5: Results

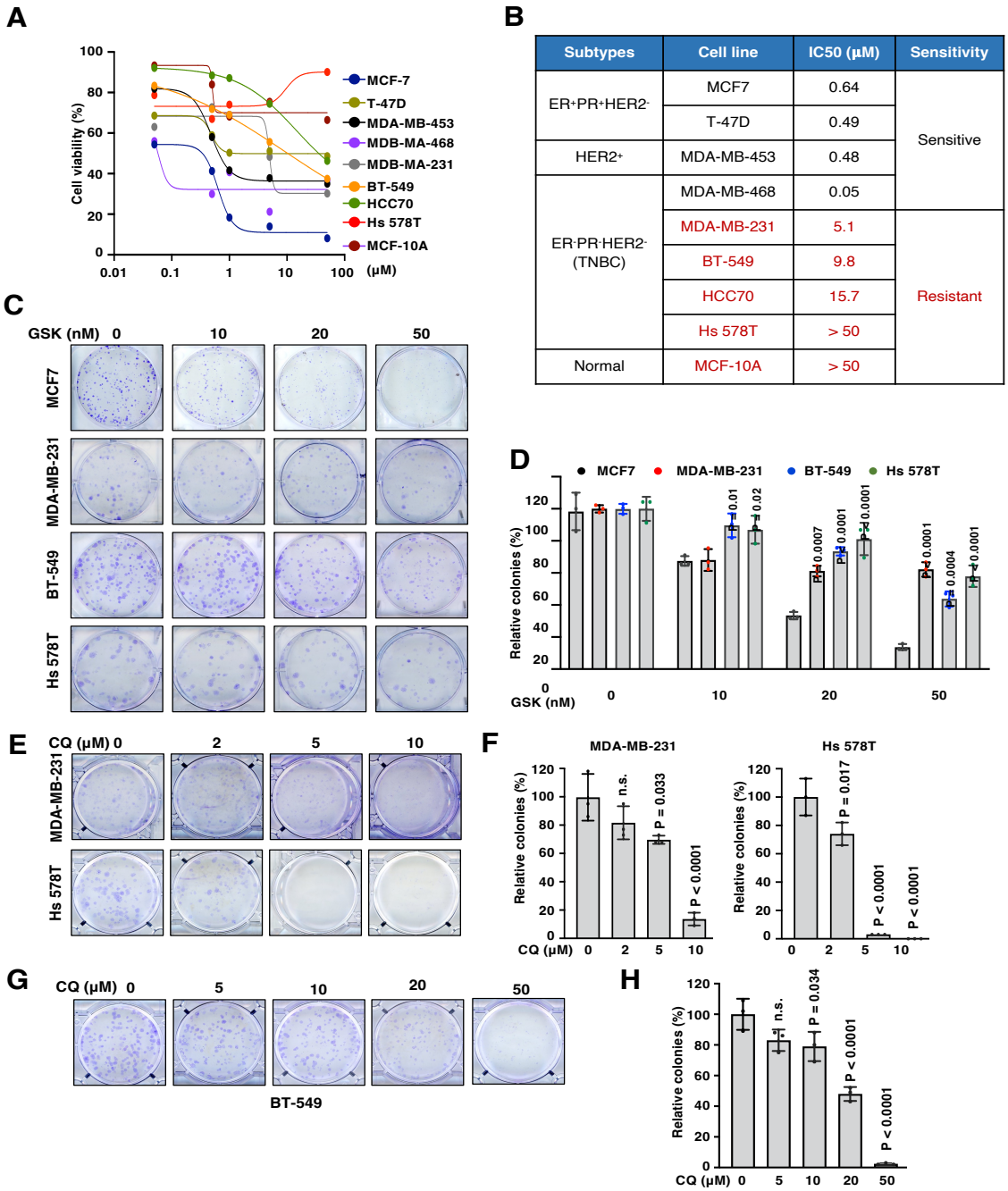
This chapter has been published from the following publication:

Brobbey, C., Yin, S., Liu, L. et al. Autophagy dictates sensitivity to PRMT5 inhibitor in breast cancer. Sci Rep 13, 10752 (2023).

5.1 Inhibiting Autophagy Enhances Sensitivity to PRMT5 Inhibitors in Breast Cancer Cells

Several clinical trials are evaluating the safety, pharmacokinetics, and pharmacodynamics of PRMT5 inhibitors in solid and hematologic tumors. Notably, one inhibitor, GSK3326595 has undergone a phase II trial for breast cancer (NCT04676516). Importantly, if this trial will be effective or if resistance will emerge against the background that PRMT5 inhibitors takes longer time to suppress tumor growth *in vitro* is unknown. In attempt to answer this question we tested sensitivity of GSK3326595 across different breast cancer cells. We found out that ER+PR+ and HER2+ breast cancer cells, and one TNBC cell line (MDA-MB-468) were sensitive to GSK3326595, whereas the other four TNBC cell lines and a widely used non-malignant breast epithelial cell line (MCF10A) were relatively resistant to GSK3326595 (**Figure 5.1.1 A and B**) with cells of IC50 $< 4\mu M$ defined as sensitive while those with IC50 $> 4\mu M$ were defined as resistant as previously described[4]. We also performed colony formation assay to confirm this resistant phenotype in TNBC cells (**Figure 5.1.1 C and D**). Based on this result, we ruminated that understanding underlying cause of relative resistance in TNBC cells will provide therapeutic window for effective treatment.

Autophagy is a known survival and protective mechanism that has been documented to protect breast cancer cells from deleterious stresses like chemotherapy and targeted therapy[282,283]. We predicted that autophagy might be providing protection under this stress condition leading to the resistance. To interrogate this claim, we treated TNBC cells with GSK3326595 and CQ, the only FDA-approved autophagy inhibitor that functions by preventing lysosomal degradation[220]. We first established cell line-specific doses that were used in the combination treatment by treating cells with different doses of CQ using cell viability and colony formation assays. We observed that CQ treatment increased cell death and colony formation in a concentration dependent manner (**Figure 5.1.1 E – H**). Interestingly, we observed differential sensitivity to CQ among the TNBC cells with Hs 578T cells being most sensitive. Having established the cell specific dose for CQ, we performed combination treatment with GSK3326595 and CQ and evaluated how CQ affected the antiproliferative effective of PRMT5 inhibitors. A combination of GSK3326595 with CQ significantly reduced colony formation across all TNBC tested, compared to single agents (**Figure 5.1.2 A – D**). We also blocked autophagy by genetic deletion of Beclin 1 which is critical for nucleation stage of autophagy[284]. In line with the CQ treatment, we found deletion of Beclin 1 enhanced GSK3326595-induced cell death (**Figure 5.1.2 E and F**). Since PRMT5 inhibitors suppress cell proliferation in part by promoting apoptosis[285], we found that co-treatment of GSK3326595 and CQ led to significant increase of cleaved caspase 3 activation (**Figure 5.1.2 G**), one of the best-known apoptotic markers[286]. These results demonstrate that genetic and chemical blockage of autophagy sensitizes resistant TNBC cells to PRMT5 inhibitor.



Subtypes	Cell line	IC50 (μM)	Sensitivity
ER+PR+HER2-	MCF7	0.64	Sensitive
	T-47D	0.49	
HER2+	MDA-MB-453	0.48	
ER-PR-HER2- (TNBC)	MDA-MB-468	0.05	
	MDA-MB-231	5.1	Resistant
	BT-549	9.8	
	HCC70	15.7	
	Hs 578T	> 50	
Normal	MCF-10A	> 50	

Figure 5.1.1. Breast cancer cells exhibit variable sensitivity to PRMT5 inhibitor and CQ. **A, B** IC50 of various breast cancer cell lines determined by cell viability assays. Cells were treated with GSK3326595 at 0, 50 nM, 500 nM, 1 μM, 5 μM, and 50 μM for 4 days before measuring cell viability. **C, D** indicated Cells were treated with GSK3326595 (GSK) at indicated doses and subjected to colony formation assays. Representative images are shown in (**C**) with relative colony numbers and statistics shown in (**D**). **E** – **H** MDA-MB-231, HS-578T and BT-549 cell treated with escalating doses of CQ followed by colony formation assay. **E, G** show representative images and **F, H** show statistics of relative colonies.

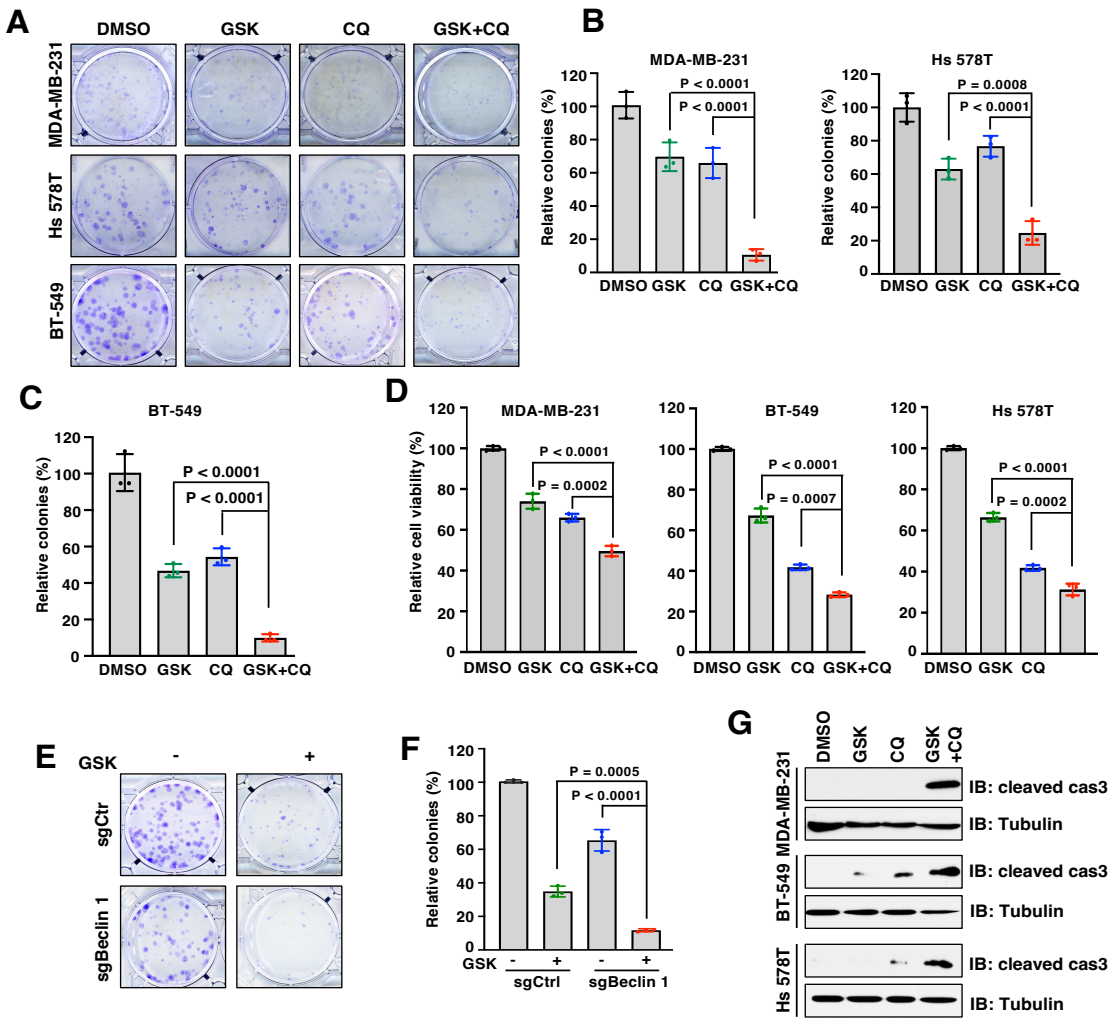


Figure 5.1.2. Autophagy inhibition sensitizes breast cancer cells to PRMT5 inhibitor.
A-C MDA-MB-231, Hs 578T and BT-549 Cells were treated with DMSO, 50nM GSK and 5 μM, 2 μM and 20 μM CQ chloroquine (CQ) respectively, or both for 8 days and 12 days respectively, and subjected to colony formation assays. Representative images are shown in (**A**), and relative colony numbers are plotted in (**B, C**). **D** Cell viability of MDA-MB-231 , BT-549 , and Hs 578T cells after treatment with GSK3326595 (GSK) and chloroquine (CQ). **E, F** BT-549 cells were knockout of Beclin 1 by sgRNA (sgCtr as a control) and then treated with 50 nM GSK for 8 days and subjected to colony formation assays. Representative images are shown in (**E**), and relative colony numbers are plotted in (**F**). In **B**, **C**, **D, F** data are shown as the mean ± SD of n = 3 independent experiments. P values were calculated by two-tailed Student's *t*-test. **G** Immunoblot (IB) analysis of whole cell lysates (WCL) derived from indicated cells treated with GSK and CQ.

5.2 Inhibition and Depletion of PRMT5 Induce Autophagy

We have shown that genetic or chemical blockage of autophagy significantly augmented antitumor effect of GSK3326595 among TNBC cells. Thus, it is plausible to assume that PRMT5 inhibitors induce autophagy that offer protection to cells from death. We explored this scenario by knocking out PRMT5 using CRISPR/Cas9 gene editing in multiple breast cancer lines and evaluated autophagy activity using accumulation of LC3-II and degradation of adaptor protein p62 as markers of autophagy induction[214]. Significantly, depletion of PRMT5 led to an elevation of LC3-II/I ratio and a reduction of p62 protein levels under normal culture condition (**Figure 5.2 A and B**). We also treated cells with escalating concentration of GSK3326595 and observed the same results (**Figure 5.2 C and D**). To corroborate these results, we used GFP-LC3 system to visually monitor the accumulation of LC3 puncta upon autophagy induction. Consistently, we observed increase LC3 puncta upon GSK treatment among different TNBC cells (**Figure 5.2 E and F**).

To decipher if the enzymatic activities of PRMT5 is indispensable for its mediated autophagy regulation, we generated stable cells expressing both the wild type and enzyme dead E444Q mutant[37], Consistently, the enzyme-dead mutant enhanced LC3-II accumulaton compared to the wild type PRMT5 (**Figure 5.2 G**), suggesting PRMT5 regulate autophagy in a enzyme dependent manner. Since autophagy is an adaptive biological process to various stress events such as nutrient deprivation, we investigated whether PRMT5 is also involved in stress-induced autophagy. To this end, we subjected PRMT5 knockout out cells to amino acid starvation and found out that compared to control cells, PRMT5 depleted cells displayed an additive LC3-II accumulation in response to amino

acids starvation (**Figure 5.2 H**). On the other hand, overexpression of the PRMT5-E444Q mutant enhanced autophagy in the absence of amino acids, compared to overexpression of PRMT5-WT (**Figure 5.2 I**). It is reported that mTORC1 phosphorylate ULK1 at S757 to inhibit its autophagy function under nutrient replete conditions[255,256]. Interestingly, we did not observe a significant difference on phosphorylation of ULK1-S757 between PRMT5-WT and PRMT5-E444Q expressing cells. These results indicate that PRMT5-mediated regulation of autophagy is likely independent of the mTORC1 pathway and has an additive effect on nutrient deficiency-induced autophagy. At this point it was abundantly clear that deletion of PRMT5 accumulate LC3-II; however, whether this due to enhanced LC3-I to LC3-II conversion or aberrant LC3-II degradation was unanswered. Of note, the accumulation of LC3-II in PRMT5-deficient cells could be caused by either enhanced LC3-I conversion to LC3-II or impaired LC3-II degradation[214,287]. To distinguish between these two possible scenarios, we measured the autophagic flux using the mRFP-GFP-LC3 reporter system, which is based on the principle that GFP, but not mRFP, is quenched in the acidic environment, such as lysosome[288]. An increase of yellow (RFP+/GFP+) and red (RFP+) puncta indicates enhanced autophagosome formation, while only accumulation of yellow puncta suggests impairment in autophagosome-lysosome fusion and degradation. Notably, we observed significant accumulation of both yellow and red puncta of LC3 in PRMT5-depleted cells (**Figure 5.2 J and K**). Moreover, treatment of cells with chloroquine, which inhibits autophagic flux by blocking autophagosome-lysosome fusion[220] further increased accumulation of LC3-II in PRMT5-depleted cells (**Figure 5.2 L**). Based on these

results suggest we concluded that PRMT5 suppresses autophagosome formation, but not autophagosome-lysosome fusion.

5.3 PRMT5 Regulates Autophagy through ULK1

We have established that PRMT5 suppress autophagy under normal conditions. We then proceeded to determine if PRMT5-mediated autophagy is via the canonical autophagy pathway involving autophagy related proteins. We reasoned that if this is the case then depletion of key players (**Figure 5.3 A**) of autophagy pathway should block LC3-II accumulation. Strikingly, depletion of ULK1, which is involved in the initiation stage of autophagy, largely blocked the induction of LC3-II in GSK3326595-treated or PRMT5-depleted cells (**Figure 5.3 B and C**). We also deleted Beclin 1, a key ATG involved in the nucleation stage of autophagy and observed the same phenotype (**Figure 5.3 D and E**). We also confirmed the immunoblot using GFP-LC3 and found out that depletion of ULK1 greatly reduced the GFP-LC3 puncta compared to control cells (**Figure 5.3 F and G**)**.** Thus, the above result is consistent with PRMT5 regulating canonical autophagy in at least through ULK1.

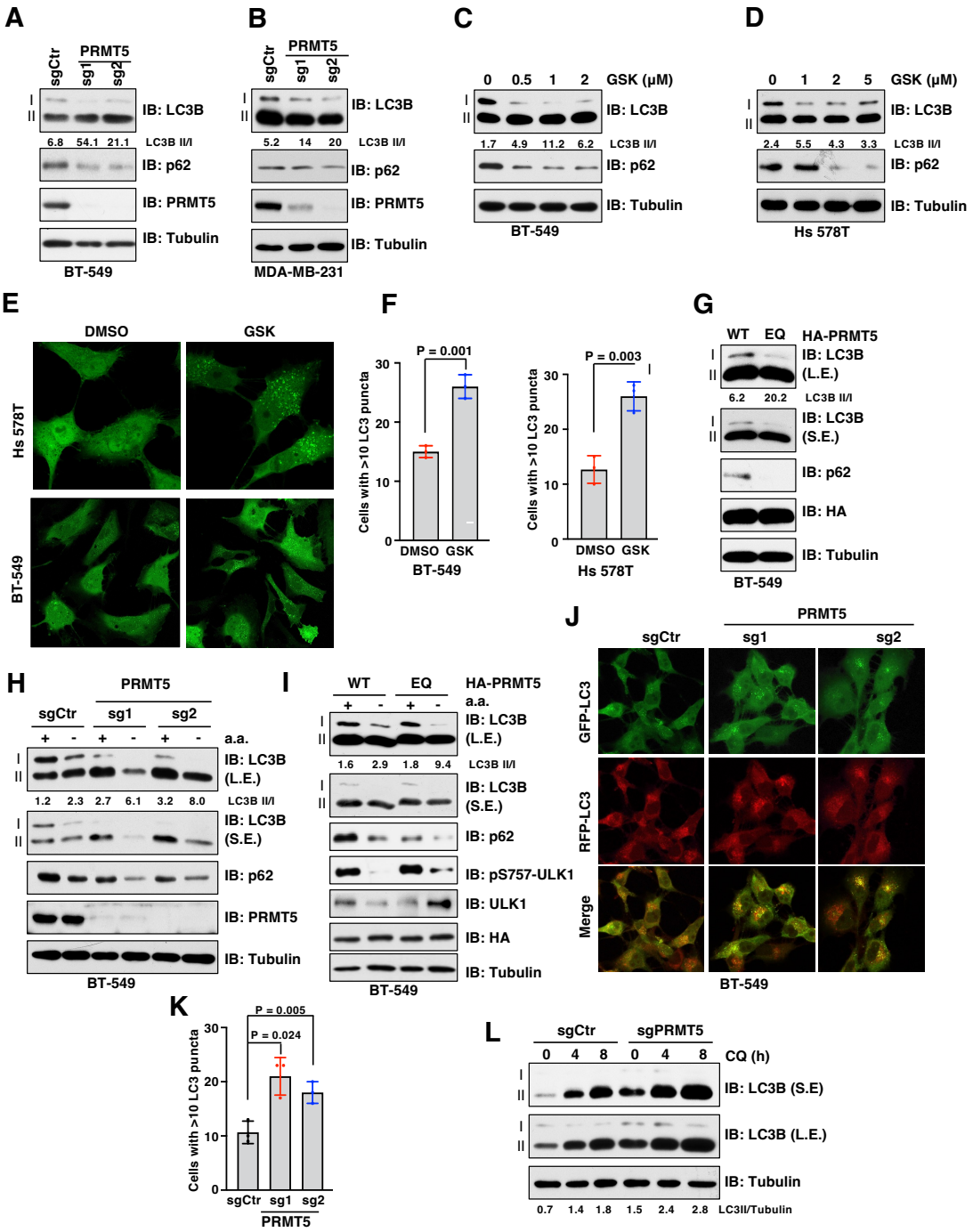


Figure 5.2. Depletion or inhibition of PRMT5 induces autophagy. A, B Immunoblot (IB) analysis of BT-549 (**A)** cells and MDA-MB-231 cells (**B**) depleted of PRMT5 cells. **C, D** Whole cell lysates from BT-549 cells and HS 578T cells treated with increasing concentration of GSK for three days. **E,F** Representative images of GFP-LC3 puncta in BT-549 and Hs 578T cells treated with DMSO or GSK for 3 days (**E**). Cells with 10 or more puncta were counted as positive and data are shown as mean ± SD of n = 3 independent experiments with a total of 50 cells counted per experiment(**F**). **G** IB analysis of WCL derived from BT-549

stably expressing PRMT5-WT or PRMT5-E444Q mutant. **H** IB analysis of WCL derived from BT-549 cells infected with sgPRMT5 and sgCtr virus. Cells were either starved of amino acids (a.a) for 4 hours or maintained in normal medium before harvesting. **I** IB analysis of WCL derived from BT-549 stably expressing PRMT5-WT or PRMT5-E444Q mutant. Cells were either starved of amino acids (a.a) for 4 hours or maintained in normal medium before harvesting. **J, K** Representative images of GFP-LC3-RFP puncta in BT-549 depleted of PRMT5 (**J**). Cells with 10 or more puncta were counted as positive and data are shown as mean ± SD of n = 3 independent experiments with a total of 100 cells counted per experiment (**K**). P values were calculated by two-tailed Student's *t*-test. **L** IB analysis of WCL derived from BT-549 cells depleted of PRMT5 and treated with 40 μM chloroquine (CQ) for 0, 4, 8 hours before harvesting.

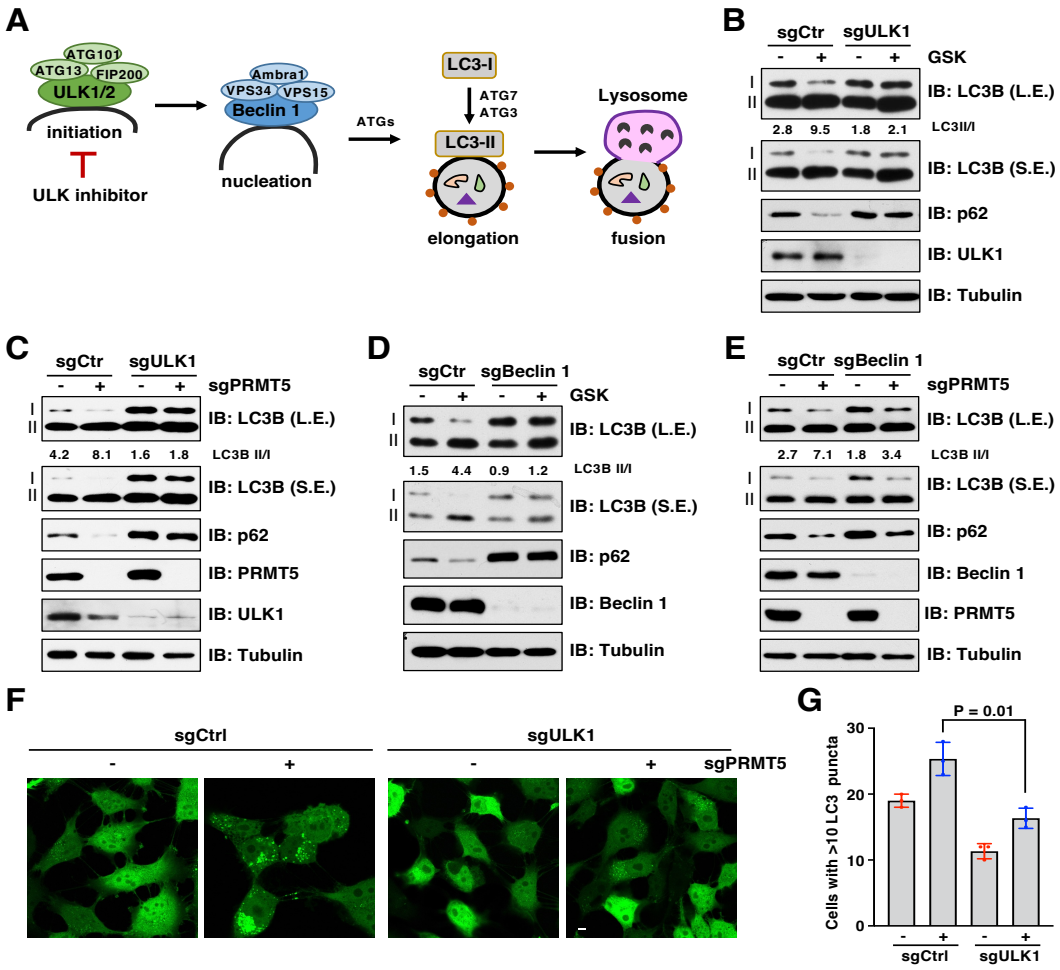


Figure 5.3. Depletion of key ATG proteins blocks PRMT5 inhibition-induced autophagy. **A** Schematic summary of the autophagy process highlighting **ULK1** and **Beclin 1** as key components in the pathway. **B** IB analysis of WCL derived from BT-549 cells depleted of ULK1. Cells were treated with GSK3326595 (GSK) for 3 days before harvesting. **C** IB analysis of WCL derived from BT-549 cells with genetic knockout of ULK1 and/or PRMT5. **D** IB analysis of WCL derived from BT-549 cells depleted of Beclin 1 by sgRNAs. Cells were treated with GSK3326595 (GSK) for 3 days before harvesting. **E** IB analysis of WCL derived from BT-549 cells depleted of Beclin 1 and/or PRMT5 by sgRNAs. **F,G** Representative images of GFP-LC3 puncta in BT-549 cells depleted of ULK1 and/or PRMT5 by sgRNAs (**F**). Cells with 10 or more puncta were counted as positive and data are shown as mean ± SD of n = 3 independent experiments with a total of 50 cells counted per experiment (**G**). P values were calculated by two-tailed Student's *t*-test.

5.4 PRMT5 Interacts with ULK1

A recent study investigating ULK1 interactome identified PRMT5 as one of the interacting proteins[13]. We, therefore, predicted that ULK1 might be a PRMT5 putative substrate in regulating autophagy. In agreement with this proteomic study, endogenous PRMT5 immunoprecipitated ULK1 but not Beclin 1 (**Figure 5.4 A**). In the reverse, ULK1 immunoprecipitated PRMT5 but no PRMT1 (**Figure 5.4 B**). We also found that ectopic ULK1 immunoprecipitated PRMT5 but not PRMT1 nor PRMT7 (**Figure 5.4 C**). Next, we investigated which domain of ULK1 is responsible for this interaction. ULK1 has N-terminal kinase domain (KD), an intrinsic disordered region that harbors several phosphorylation sites, and C-terminus responsible for interacting with ATG13, FIP200 and ATG101**.** By co-expressing ULK1 domains with PRMT5, we found that the KD is responsible for PRMT5-ULK1 interaction, deletion of which abrogates this interaction (**Figure 5.4 E and F**). PTMs such as phosphorylation by mTORC1 and AMPK[254,256], as well as acetylation by TIP60[260] under varying stress condition regulate ULK1 activity during autophagy. Interestingly, amino acid starvation induced-autophagy, which is highly dependent on ULK1[268], did not alter ULK1 interaction with PRMT5 (**Figure 5.4 G**).

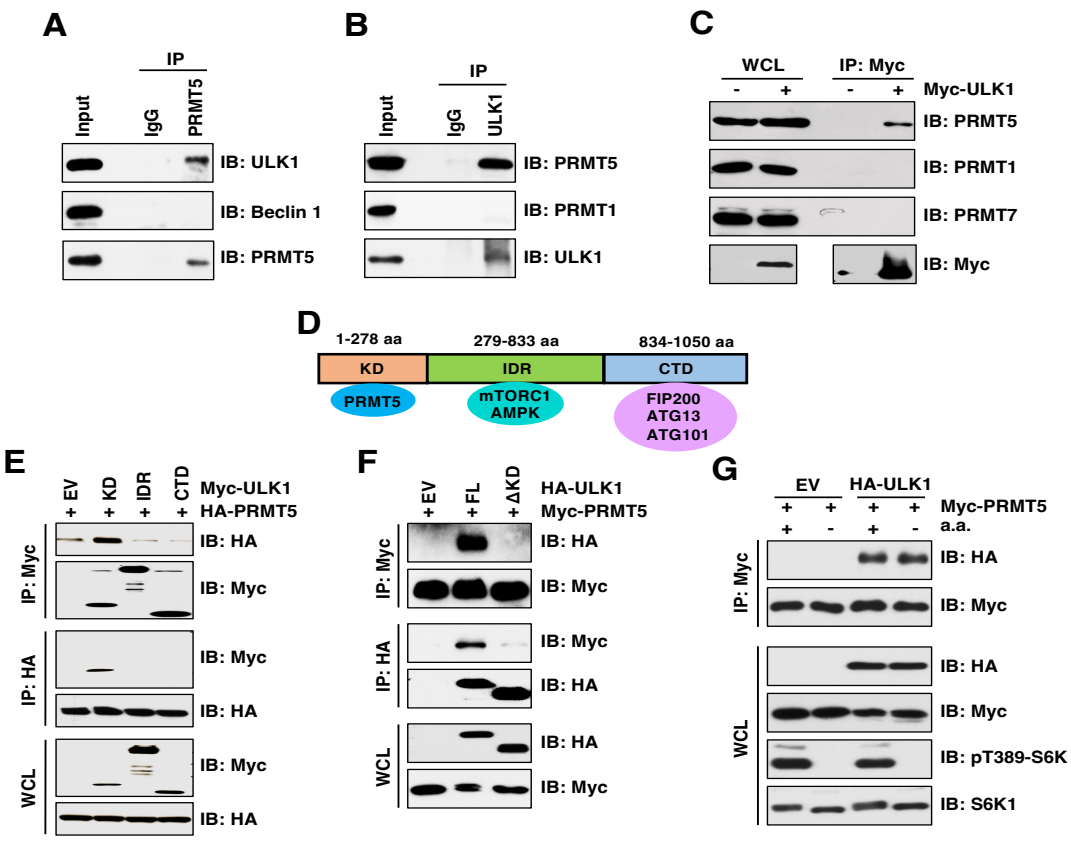


Figure 5.4. PRMT5 interacts with ULK1. A, B IB analysis of WCL and PRMT5 immunoprecipitation (IP) product derived from MDA-MB-231 cells using IgG was as negative control. **C** IB analysis of WCL and immunoprecipitation (IP) products derived from HEK293T cells transfected with Myc-ULK1. **D** Schematic summary of the ULK domains and their interacting proteins **E**, **F** IB analysis of WCL and IP products derived from HEK293T cells transfected with PRMT5 and indicated ULK1 constructs. **G** IB analysis of WCL and IP products derived from HEK293T cells transfected with indicated constructs. Cells were either starved of amino acids (a.a) for 4 hours or maintained in normal medium before harvesting.

5.5 PRMT5 Methylates ULK1 at Arginine 532

We then investigated the ramification of PRMT5-ULK1 interaction by checking if ULK1 is methylated. Immunoblot analysis showed that ULK1 is monomethylated (**Figure 5.5 A**) using pan MMA antibody[53]. To establish PRMT5 as methyltransferase of ULK1, we co-expressed PRMT5-WT or enzyme dead PRMT5-E444Q mutant with ULK1 in the presence of MEP50. Overexpression of PRMT5-WT, but not PRMT5-E444Q, enhanced ULK1 MMA signal (**Figure 5.5 B**). Further, knockout of PRMT5 dramatically reduced MMA levels of ULK1 (**Figure 5.5 C**). To identify which arginine residue is methylated in ULK1, we resorted to a prediction tool, GPS-MS[289] that analyzes protein sequence and assigns scores to potential methylated arginines. We did immunoblot analysis on six arginine residues that were top ranked after mutating the arginine residues to lysine (**Figure 5.5 D**). Notably, it was R532K but not the rest that completely blocked the ULK1-MMA signal (**Figure 5.5 E**). To provide direct evidence that PRMT5 is directly responsible for ULK1-R532 methylation, we performed *in in vitro* methylation assay with truncated GST-ULK1 protein purified from bacteria as the substrate and PRMT5/MEP50 immunoprecipitated from cells as the methyltransferase in the presence of radioactive SAM. As expected, ULK1- WT, but not the ULK1-R532K, was methylated by PRMT5 (**Figure 5.5 F**). Consistent with the *in vitro* results, overexpression of PRMT/MEP50 could not methylate ULK1-R532K (**Figure 5.5 G**). Though PRMT5 is the predominant SDMA PRMT, we could not detect ULK1-SDMA using pan-SDMA antibody[53]. However, we could not rule out SMDA modification of ULK1-R532 because the pan-SDMA is not optimized to detect this modification on ULK1. Indeed, our mass spectrometry data indicates that ULK1-R532 has SDMA modification (**Figure 5.5 H**).

In line with ULK1-PRMT5 interaction not changing under amino acid starvation (**Figure 5.4 G**), its monomethylation signal remained unchanged under amino acid-deficient condition (**Figure 5.5 I**). Based on these results, we concluded that PRMT5 methylates ULK1 on arginine 532, which is likely independent of major upstream regulators of ULK1.

5.6 ULK1-R532 Methylation Decreases Its Kinase Activity

Having provided evidence that ULK1 is methylated by PRMT5, we investigated how this methylation affects its kinase activity and autophagic function. During autophagy process when ULK1 is activated, it phosphorylates several downstream targets to drive autophagy. For example, ULK1 phosphorylates Beclin 1 at S15[258] and ATG13[259] at S318 to initiate autophagy. We evaluated the effect of ULK1-R532 methylation by testing its kinase activity *in vitro* using Beclin 1 S15 as substrate. ULK1-R52K enhanced phosphorylation of Beclin 1 S15 compared to ULK1-WT (**Figure 5.6 A**). Consistently, ULK1-R532K increased phosphorylation of Beclin1 S15 and ATG13 S318 in cells compared to ULK1-WT (**Figure 5.6 B–D**). In line with the enhanced activity of ULK1-R532K, cells expressing ULK1-R532K mutant exhibited an increased ratio of LC3-II/I and degradation of p62, compared to cells expressing ULK1-WT (**Figure 5.6 E**). These results suggest that PRMT5-mdediated ULK1-R532 methylation decreases its kinase activity to attenuate autophagy.

Next, we sought to investigate how ULK1-R532K enhances its kinase activity. We evaluated whether ULK1 methylation affects ULK1 complex assembly. Both ULK1-WT and ULK1-R532K bound FIP200 and ATG13 at a comparable level (**Figure 5.6 F and G**), indicating the methylation does not affect ULK complex formation. Similarly, no significant

changes were observed for ULK1-R532K binding to its substrates Beclin 1 and Ambra 1 (**Figure 5.6 H and I**) . Moreover, the interaction between ULK1-R532K and AMPK or Raptor (an essential subunit of mTORC1) was not significantly changed, compared to ULK1-WT (**Figure 5.6 J**), further supporting the notion that R532 methylation regulates ULK1 activation independent of or parallel to the mTORC1/AMPK pathway. These results suggest that ULK1-R532 methylation impairs its kinase activity unlikely through modulating ULK1 interactions with its binding partners.

5.7 ULK1 Inhibitor Sensitizes TNBC Cells to PRMT5 Inhibitor

Since ULK1 is a key druggable serine/threonine kinase for the induction of cytoprotective autophagy, targeting ULK1 therefore represents a promising therapeutic strategy for overcoming drug resistance[290]. Having demonstrated that ULK1 plays a critical role in PRMT5-mediated autophagy regulation, we interrogated whether ULK1 inhibition would enhance sensitivity to PRMT5 inhibitor. We first asked if inhibiting ULK1 can suppress PRMT5 deletion or inhibition induced autophagy. Indeed, treatment with ULK1/2 inhibitor MRT68921 largely suppressed GSK3326595-induced autophagy, as evidenced by a decrease of the LC3B II/I ratio and GFP-LC3B puncta (**Figure 5.7 A–C**). This result suggests that inhibiting ULK1 can block the cytoprotective autophagy and potentially eradicate resistance to PRMT5 inhibitors. In agreement with this preposition, combination of MRT68921 with GSK3326595 significantly decreased cell viability and colony formation in TNBC cells, compared to single agent (**Figure 5.7 D–H**).

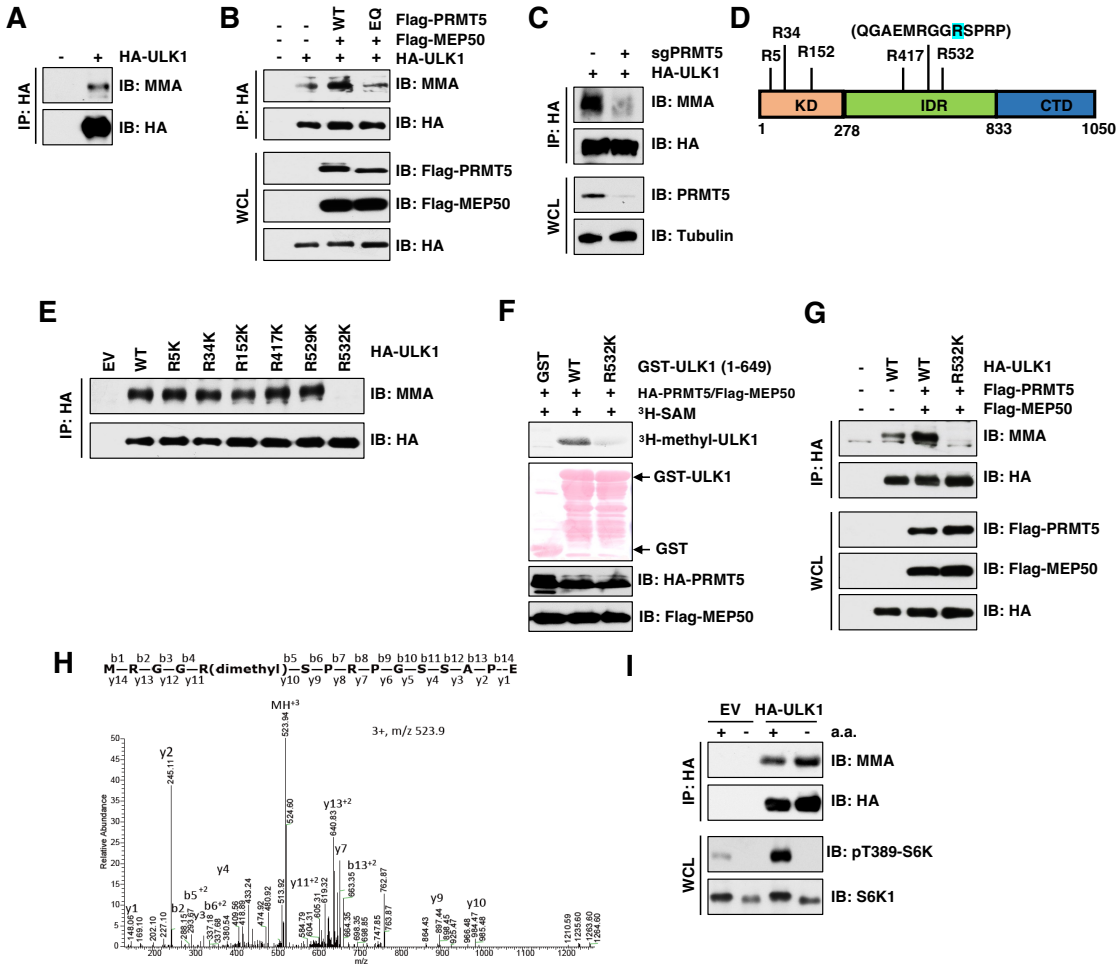


Figure 5.5. PRMT5 methylates ULK1 at Arg532. A IB analysis of IP products derived from HEK293T cells transfected with HA-ULK1. **B** IB analysis of WCL and IP products derived from HEK293T cells transfected with indicated constructs. **C** IB analysis of WCL and IP products derived from MDA-MB-231 cells stably expressing HA tagged ULK1 and infected with sgPRMT5 or sgCtr virus. **D** Schematic presentation of the putative methylated residues of ULK1. **E** IB analysis of WCL and IP products derived from HEK293T cells transfected with indicated constructs. **F** In vitro methylation of ULK1 in the presence of ^{3}H-SAM. GST-ULK1 (1-649 a.a) was purified from bacteria and HA-PRMT5 were immunoprecipitated from HEK293T cells. **G** IB analysis of WCL and IP products derived from HEK293T cells transfected with indicated constructs. **H** Mass spectrometry analysis of ULK1-Arg532 methylation **I** IB analysis of WCL and IP products derived from HEK293T cells transfected with indicated constructs. Cells were either starved of amino acids (a.a) for 4 hours or maintained in normal medium before harvesting.

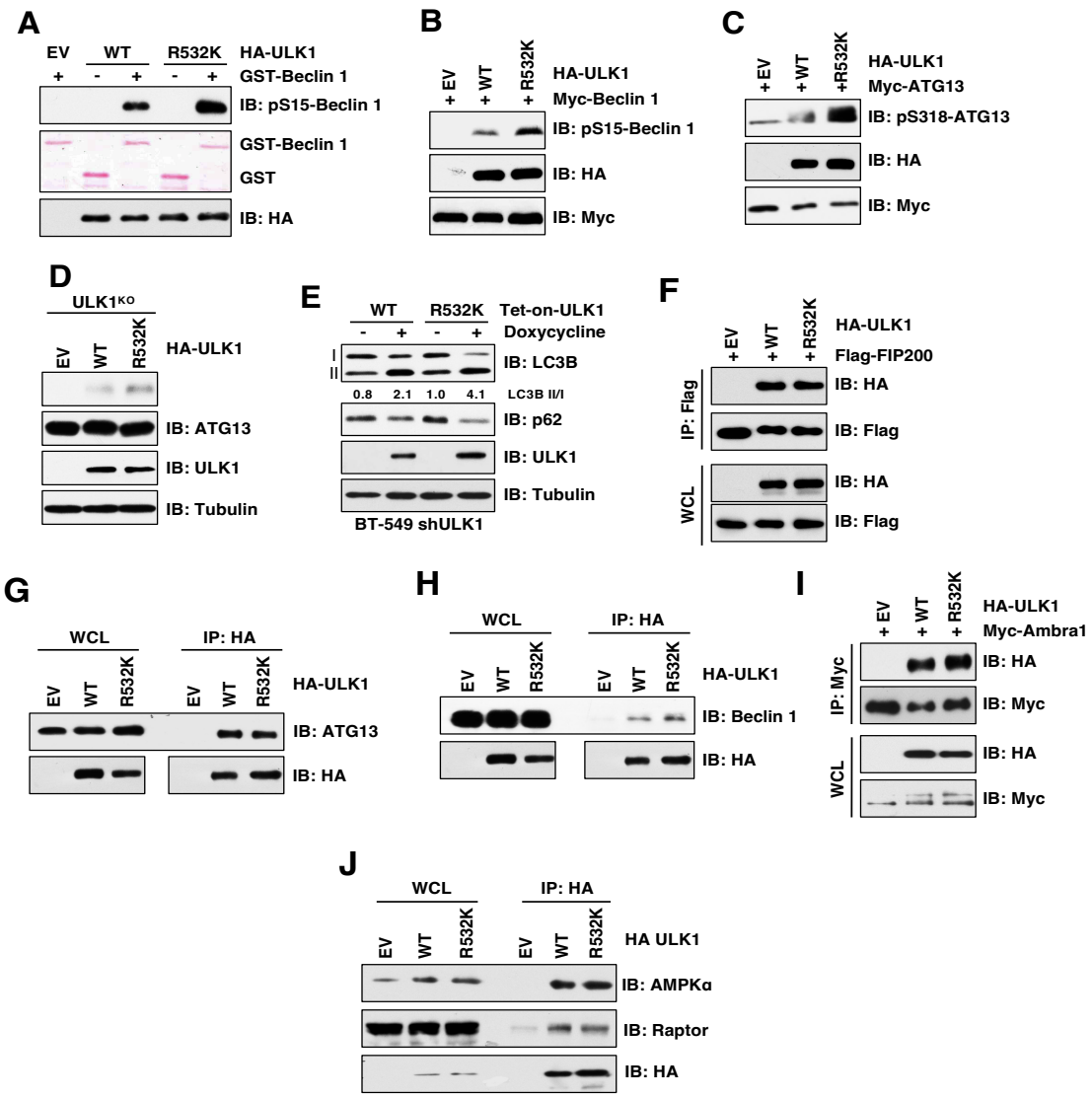


Figure 5.6. Methylation of ULK1 at Arg532 suppresses its kinase and autophagic activity. **A** In vitro ULK1 kinase assay was performed using recombinant GST-Beclin1 (1–86 aa) purified from bacteria and HA-ULK1 immunoprecipitated from HEK293T cells. **B, C** IB analysis of WCL derived from HEK293T cells co-transfected with ULK1 and Beclin 1 (**B**) or ATG13 (**C**). **D** IB analysis of WCL derived from ULK1-knockout (KO) MDA-MB-231 cells stably expressing ULK1-WT or ULK1-R532K. **E** IB analysis of WCL derived from BT-549 cells depleted of endogenous ULK1 and re-expressing doxycycline inducible ULK1-WT or ULK1-R532K. Cells were treated with 1 μg/ml doxycycline for 8 h before harvesting. **F–J** IB analysis of WCL and IP produces derived from HEK293T cells transfected with indicated protein constructs.

Moreover, apoptosis was strongly enhanced in cells treated with both GSK3326595 and MRT68921, compared to cells treated with single agent (**Figure 5.7 I**). Since ULK1-R32K has high basal autophagic function than ULK1-WT type, we predicted the former will have relative survival advantage upon PRMT5 inhibition. Truly, cells expressing ULK-R532K displayed more colonies than cells expressing ULK-WT in the presence of GSK3326595 (**Figure 5.7 J and K**). These results suggest that ULK1 inhibition suppresses cytoprotective autophagy and consequently confers sensitivity to PRMT5 inhibitor in TNBC cells.

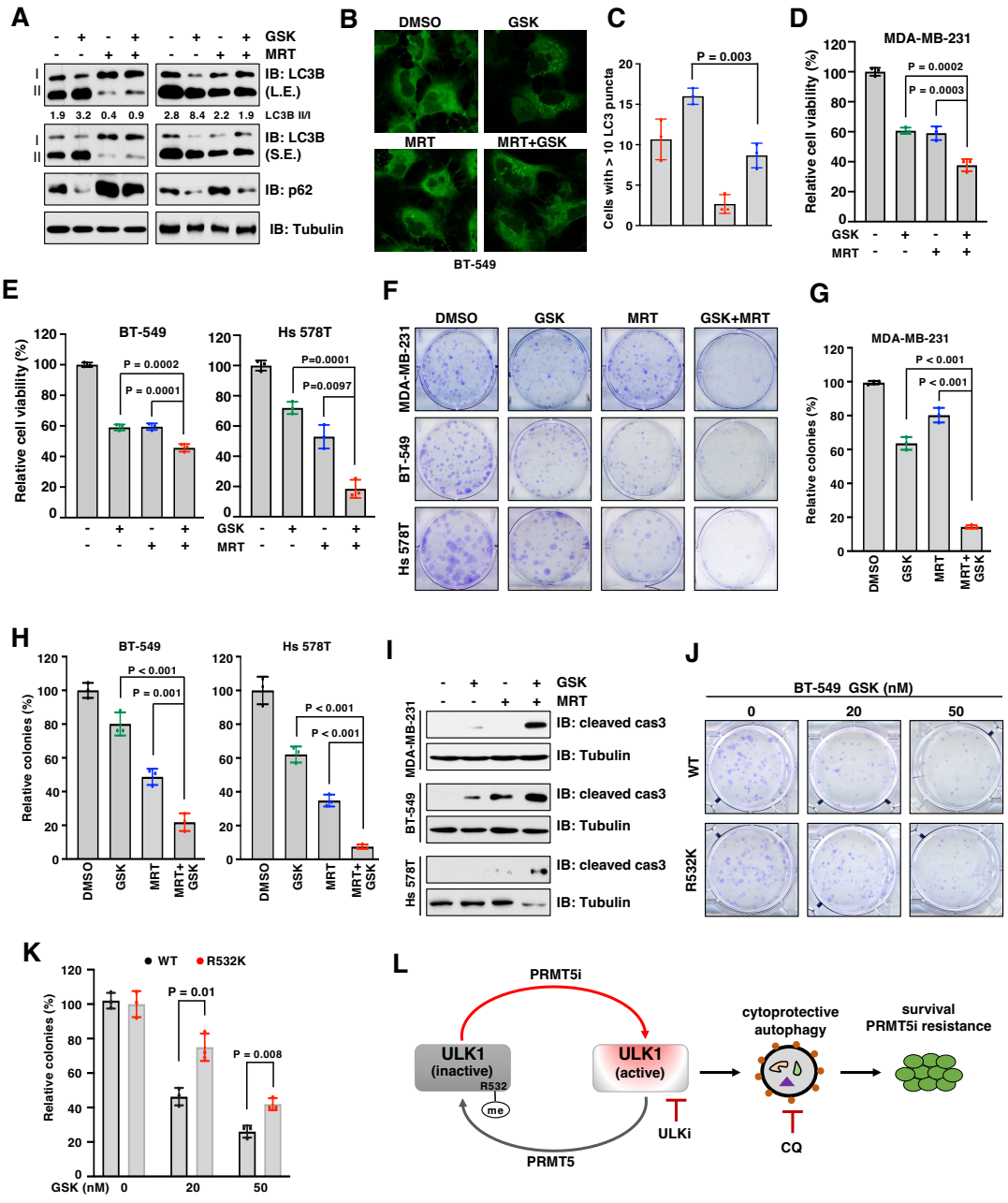


Figure 5.7. ULK1 inhibitor enhances cellular sensitivity to PRMT5 inhibitor. A IB analysis of WCL derived from indicated cells treated with 1 µM GSK3326595 (GSK) and 300 nM, 1.5 µM, and 0.5 µM MRT68921 (MRT, ULK inhibitor) for MDA-MB-231, BT-549, and Hs 578T respectively for 3 days before harvesting. **B,C** Representative images of GFP-LC3 puncta in BT-549 cells treated with 1µM GSK or 1µM MRT or both for 3 days **(B).** Scale bar, 25 µM B. Cells with 10 or more puncta were counted as positive and data are shown as mean ± SD of n = 3 independent experiments with a total of 50 cells counted per experiment (**C**). P values were calculated by two-tailed Student's t-test. **D,E** Cell viability of MDA-MB-231, BT-549, and Hs 578T cells after treatment with 1 µM GSK and 300 nM,

1.5 µM, and 0.5 µM MRT, respectively, for 4 days (MDA-MB-231 and BT-549) and 6 days (Hs 578T). **F** – **H** MDA-MB-231, BT-549 and Hs 578T cells were treated with DMSO,100 nM, 15 nM, and 50 nM GSK, respectively, and MRT concentration as described in **D,E**. **I** IB analysis of WCL derived from indicated cells after treatment with 1 µM GSK and MRT as described in **D,E** for 3 days. **J,K** BT-549 cells were depleted of endogenous ULK1 and re-introduced inducible ULK1-WT or R532K. The resulting cells were treated with 0, 20, and 50 nM GSK and subjected to colony formation assays. **L** Graphical overview of how PRMT5 regulates autophagy by methylating ULK1 and how targeting autophagy overcome resistance to PRMT5 inhibitor. PRMT5i, PRMT5 inhibitor; ULK1i, ULK1 inhibitor; CQ, chloroquine. In **D,E,G,H** data are shown as the mean ± SD of n = 3 independent experiments. P values were calculated by Student's t test. Similar results were obtained in n = 3 independent experiments in **A, I**.

Chapter 6: Discussion

PRMT5 was first characterized as a transcriptional repressor by catalyzing symmetric dimethylation of histone H3 arginine 8 (H3R8) and H4 arginine 3 (H4R3), leading to transcriptional repression of tumor suppressor genes[110-112]. As an oncoprotein whose overexpression has been implicated in severity and poor prognosis of cancer patients, several inhibitors aimed at counteracting PRMT5-mediated tumorigenesis have been generated with the highly potent and specific GSK3326595 being used different at stages of clinical trials[291]. Previous studies have shown variation in sensitivity of breast cancer cells to PRMT5 inhibitors with luminal A breast cancer type like MCF7 being sensitive while TNBC like BT-549 ,MDA-MB-231, Hs-578T are the most resistant[4,168], but the cause of this variable sensitivity has not been addressed. One of the banes of cancer treatment is the toxicity associated with monotherapy. Indeed, Immune system suppression and impaired hematopoiesis are some of the side effects of PRMT5 inhibition[189]. As a result, combinational therapy has been an attractive strategy to mitigate toxicities while simultaneously improving efficacy of inhibitors. For the first time we show that depletion or inhibition of PRMT5 evokes autophagy in different subtypes of BC cells where the autophagy offers protection against cells death. Our data shows that knockout of PRMT5 induces autophagy evidenced by accumulation of LC3-II on western blot as well as increased GFP and RFP puncta. These findings suggest that under normal condition, PRMT5 suppresses autophagy, revealing another cellular process regulated by PRMT5. Importantly, blocking autophagy with CQ or depletion of Beclin 1 increases sensitivity to PRMT5 inhibitor to amplify its antitumor effect. Interestingly, blocking autophagy has

yielded similar results with tamoxifen and endoplasmic reticulum stress aggravators in BC cells[292,293], corroborating the cytoprotective functions of autophagy. Given that PRMT5 inhibitor induces cytoprotective autophagy, it is safe to suggest that relative resistance to PRMT5 inhibitor observed in Hs 578T, BT-549 and MDA-MB-231 cells is in part due to high residual autophagy that cushions them against cytotoxicity of PRMT5 inhibitors. Our data reiterate the efficacy of combinational therapy and paves way for treatment of BC with dual PRMT5 and autophagy inhibition.

Several layers of autophagy regulation have been documented ranging from transcription to PTMs[199,217]. Though ubiquitylation, phosphorylation, and acetylation of various ATG proteins have been reported[174], data on arginine methylation of ATG proteins is unclear. However, indirect role of PRMTs in autophagy has been documented. Shin et al. reported that glucose starvation promoted PRMT4 stability due to reduced SKP2-mediated ubiquitylation and degradation. This stabilized PRMT4, in turn, induced H3R17 methylation and the expression of autophagy and lysosomal genes[294]. In *C. elegans*, the PRMT1 homolog facilitated the degradation of PGL granules during embryogenesis in autophagy-dependent manner. It was revealed that PRMT1 dimethylated PGL-1 and PGL-3, enhancing their interaction with the cargo receptors SEPA-1 and EPG-2 allowing their delivery int to autophagosome[295].

Here, we show that PRMT5 regulates autophagy by directly methylating ULK1 on arginine 532 to suppress its kinase and basal autophagic functions. ULK1 is the only conserved serine and threonine kinase among the ATGs that serves as the upstream sensor of the cellular environment to initiate autophagy. It is believed that contrary to

yeast where the ULK1 complex assembles upon starvation, in mammals ULK1 complex is constitute[245], implying other layers of regulation likely PTMs dictate the activation of the complex. Notably, ULK1 and its close homolog ULK2 are subject to manifold PTMs such as ubiquitylation and phosphorylation. For instance, it has been predicted that the intrinsic disordered regions of ULK1 harbors about forty potential phosphorylation sites[248]. Added to this, we demonstrate that arginine 532 of ULK1 which lies in the IDR of ULK1 is methylated by PRMT5 and this methylation is independent of classical upstream regulators. Reconstituting ULK-R532K in a ULK1 knockout cells elicited a relatively strong autophagic functions compared with the wild type with concomitant increase in LC3-II accumulation and increased phosphorylation of ATG13. This was further supported by *in vitro kinase* experiments. Interestingly, we observe that ULK1-R532K does not induce much LC3-II compared to when PRMT5 was depleted. We propose two reasons to this. First, there could be other potential ATG proteins which are PRMT5 substrates. Second, PRMT5 may regulate other upstream autophagy modulators that may synergize with ULK1 methylation. For example, PRMT5 has been documented to methylate and enhance AKT activation[9], which is a negative regulator of autophagy. More so, PRMT5 regulates DNA damage repair, which is a potent inducer of autophagy[17,27].

Except for ubiquitylation that has been shown to affect ULK1 stability[264,296,297], the mechanism of how most PTMs especially phosphorylation, affect the autophagic functions of ULK1 is not clearly established. Similarly, we did not observe any palpable difference between ULK1-R532K stability and its binding to substrates and members of the complex compared to the wild type. Since methylation affects hydrogen bond

interaction that has the propensity to confer structural reorientation of proteins[18], we speculate that ULK1-R532 methylation may alter its conformation to promote clustering or dimerization of ULK1, leading to enhanced autophosphorylation or this methylation might regulate other unknown ULK1 PTMs in its IDR that modulate its kinase dynamics. For example, Ser533, Ser469, Ser495 of ULK1 has been shown to be phosphorylated by TOPK to suppress its kinase function in glioma cells[298]. Thus, solving ULK1-IDR structure could illuminate how ULK1-R532 methylation suppresses its kinase and autophagic functions.

A recent paper reported that ULK1 is symmetrically dimethylated at R170 by PRMT5 to stimulate its kinase activity under hypoxic environment[299]. However, it is unclear whether R170 is the sole site methylated by PRMT5 because they detected ULK1 arginine methylation only using the anti-ULK1-R170me2s antibody while we used radioisotope based in vitro assay to reveal R532 as the major methylation site in ULK1 by PRMT5. Moreover, ULK1-R170 methylation occurs in the KD domain to stimulate its function only in hypoxic environment while ULK-R532 occurs in the IDR region to suppress its kinase activities under normal conditions. Of note, the ULK1-R170 methylation signal remained unchanged under normal conditions upon PRMT5 depletion. Even though we did not evaluate ULK1-R532 methylation under hypoxic conditions, the fact that the methylation signal dwindled upon PRMT5 knockout and PRMT5 inhibitor treatment suggests that ULK1-R532 methylation is likely independent of hypoxia. Importantly, PRMT5 depletion did not induce nor suppress autophagy under normal conditions in their study, which is contrary to what we present in this study that PRMT5 depletion induced

autophagy. Knowing that ULK1 phosphorylation by mTORC1 at Ser757 and Ser555 suppresses and activates its kinase function, respectively, it is not surprising that arginine methylation at R532 and R170 may alter its function in opposite direction. It would be interesting to investigate if ULK1-R532 methylation would elicit autophagy under hypoxic conditions.

Most importantly, blocking ULK1 kinase function increases sensitivity of otherwise GSK resistant TNBC, implying that cytoprotective autophagy induced upon PRMT5 inhibition is in part through activation of ULK1 kinase activity. We believe this study will serve as a springboard to further interrogate the feasibility of using ULK1 inhibitors in combination with PRMT5 inhibitors in cancer treatment. Future studies could investigate if PRMT5 inhibition induces autophagy in other cancer types and if autophagy inhibitors could synergize with PRMT5 inhibitors in these cancers.

Chapter 7: Concluding Remarks

PRMT5 has gained traction as antitumor target over the years due to its oncogenic functions across slew of cancers. Though several efforts, resources and time have been invested in designing or identifying inhibitors to target it for treatment, there is still a wide knowledge gap on how it exerts its tumorigenic effects. As of today, there have been about eight clinical trials evaluating the efficacy of PRMT5 inhibitors, many of which have recorded gamut of toxicities from alopecia to pancytopenia. These toxicities have necessitated termination of some trials. Thus, it is imperative to expand the biology of PRMT5 through rigorous studies to better refine existing inhibitors or discover new ones for safe and efficacious cancer treatment.

My graduate work reported herein has made modest contribution to narrowing this knowledge gap by revealing autophagy as important cellular process controlled by PRMT5 at least in breast cancer cells. One novelty of this study is establishing that PRMT5 inhibitors induce autophagy, which we believe is a contributory cause of relative resistance in TNBC. Moreover, this study has identified ULK1 as substrate of PRMT5 in autophagy. It was my expectation that PRMT5 inhibitors and CQ or ULK1 inhibitors be combined in mice setting to further provide robust evidence of the synergy between these two inhibitors. However, I am optimistic these mice studies will be conducted in the future with an improved method for delivering GSK3326595.

One important observation in this study is that arginine methylation-deficient ULK1 (ULK1-R532K) did not elicit robust LC3-II accumulation compared to the wild type when reconstituted in ULK1-knockdown cells. This suggests that there are other potential

pathways contributing to greater autophagy induction in response to PRMT5 inhibitors. While I have a hunch that this might be due to other PRMT5 substrates or DNA damage-induced stress, I cannot exclude the possibility of other independent pathways. Resolving this puzzle in the future will be interesting, and it could be achieved by generating PRMT5 inhibitor-resistant cells through protracted treatment of sensitive cells, such as MDA-MB-468 or MCF7, with GSK3326595. I believe a comparison of RNA-seq data between resistant and sensitive cells will help identify other contributory pathways that promote autophagy induction or independent pathways responsible for resistance to GSK3326595.

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