IDENTIFICATION OF A NEW TUMOR SUPPRESSOR PATHWAY MODULATING RAPAMYCIN SENSITIVITY IN COLORECTAL CANCER

TAN JING

NATIONAL UNIVERSITY OF SINGAPORE
2011

IDENTIFICATION OF A NEW TUMOR SUPPRESSOR PATHWAY MODULATING RAPAMYCIN SENSITIVITY IN COLORECTAL CANCER

TAN JING

(MSc., Xiamen University)

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTORATE OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2011

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Professor YU Qiang, for his excellent guidance, enthusiastic encouragement and kind support during my Ph.D study. I would also like to thank my co-supervisor Professor HOOI Shing Chuan, for the guidance and constant support through the course of my study.

I would also like to express my deep appreciation to LEE Puayleng for her significant help and technical supports in the whole Ph.D project. In addition, I wish to extend my regards to Lee Shuet Theng, Feng Min, and Adrian WEE Zhen Ning for valuable advice and help in my thesis preparation. I would also like to extend my sincere appreciation to all the lab members at the laboratory of Cancer Biology and Pharmacology, Ms. Li Zhimei, Ms. Jiang xia, Ms. Aau Mei Yee, Ms. Cheryl Lim, Mr. Eric Lee, Dr. Wu Zhenlong, Dr. Wong Chew Hooi, Dr. Qiao Yuanyuan for the help.

Finally, I am heavily in debt to my family for all the love and support, especially my wife for her complete understanding all through the course of my PhD study. I would like to dedicate this thesis to my family, without whom none of this would have been possible.

This project is funded by the National University of Singapore, Genome Institute of Singapore and SSD A-STAR fellowship.

Table of Contents

Summary

Both genetic and epigenetic defects causing alterations to gene expression are implicated in cancer development. Epigenetic repression of gene transcription through DNA methylation is one of the fundamental mechanisms for inactivation of tumor suppressor genes in many cancers. Thus, identification of these silencing tumor suppressor genes could provide insight into the biological processes and pathways underlying tumorigenesis. In this thesis, we provide a comprehensive approach that integrates gene expression and ChIP-seq data for identification of DNA methylation silencing tumor suppressors and their-associated signaling pathways in colorectal cancer. A total of 203 colon cancer methylation silencing (CMS) genes have been identified and further characterized. Among the 203 CMS genes, *PPP2R2B*, one of the regulatory B subunits of protein phosphatase 2A (PP2A), was selected for further functional study.

Tumor suppressor PP2A complex is a major serine/threonine phosphatase that serves as a critical cellular regulator of cell growth, proliferation, and survival. However, how its change in human cancer confers growth advantage is largely unknown. This study shows that *PPP2R2B*, encoding the B55β regulatory subunit of PP2A complex, is epigenetically inactivated by DNA hypermethylation in most of human colorectal cancer patients. Functional studies show that *PPP2R2B* re-expression in colorectal cancer (CRC) cells resulted in senescence, decreased cell proliferation, and xenograft tumor growth inhibition. In addition, *PPP2R2B* knockdown promotes cellular transformation in immortalized human epithelial cells.

Therefore, gain- and loss-of-function data suggest that the loss of *PPP2R2B* facilitates oncogenic transformation. Mechanistically, we have demonstrated that *PPP2R2B* forms a functional PP2A complex targeting and inhibiting p70S6K and Myc phosphorylation. Taken together, our data show that *PPP2R2B*-specific PP2A complex functions as a tumor suppressor and its loss contributes to the deregulated S6K and Myc signaling, leading to growth advantage of CRC.

Furthermore, we show that *PPP2R2B*-regulated tumor suppressor pathway has a role in modulating mTOR inhibitor sensitivity. The mTOR signaling pathway plays a central role in tumor development, making this pathway as attractive target for cancer therapy. Small molecule drugs targeting mTOR, such as rapamycin, have been shown to be promising for cancer therapy. However, the clinical responses to the rapamycin as mTOR-targeted therapy are frequently confounded by acquired resistance. In colon cancer, loss of *PPP2R2B* leads to induction of PDK1-dependent Myc phosphorylation in response to rapamycin. Conversely, re-expression of *PPP2R2B* blocks the PDK1-Myc signaling, leading to re-sensitization to rapamycin. We also show that genetic ablation or pharmacologic inhibition of PDK1 abrogates rapamycin-induced Myc phosphorylation, leading to rapamycin sensitization. Thus, our data demonstrate a new mechanism underlying rapamycin resistance in CRC, which is independent of PI3K-AKT and MAPK negative feedback loops. Together, these results identified *PPP2R2B* as a new biomarker to predict the rapamycin response and also provided a new therapeutic strategy to overcome the rapamycin resistance in cancer therapy.

List of Tables

List of Figures

List of Abbreviations

Symbol	Definition
7-AAD	7-Aminoactinomycin D
APC	Adenomatosis polyposis coli
ATP	Adenosine triphosphate
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation-sequencing
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	deoxynucleotide triphosphates
DOX	Doxycycline
ECL	Enhanced chemiluminescence
EDTA	Ethylene Diamine Tetra-acetic Acid
FACS	fluorescence assisted cell sorting
FBS	fetal bovine serum
FBS	Fetal bovine serum
FDR	False discovery rate
GFP	Green fluorescent protein
HRP	Horseradish peroxidase
mRNA	messenger RNA
NaF	sodium fluoride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinyllidene difluoride
qPCR	Quantitative PCR
RT-PCR	Reverse-transcription PCR
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
siRNA	Short interference RNA
CMS	Cancer methylation silencing

1 Chapter I: Introduction

1.1 Loss of tumor suppressor genes by genetic and epigenetic alterations in cancer

Cancer is a complex disease in which the phenotypes of different types of cancers correlate with distinct genetic and epigenetic alterations. A wide range of genetic alterations, including somatic point mutations, deletions, chromosomal rearrangements and copy number changes, lead to inactivation of tumor suppressor and activation of oncogenes during cancer development. In addition to the widely observed genetic changes, epigenetic alterations are also found to play a important role in tumor progression. Gene suppression by epigenetic alteration is commonly mediated through DNA methylation and histone modification. In this section, I will briefly discuss the role of genetic and epigenetic changes leading to inactivation of tumor suppressor genes in tumor progression.

1.1.1 Genetic alterations as a cause of loss-of-function of tumor suppressor genes in cancer

Cancer is essentially a genetic disease arising from the concerted effect of multiple genetic changes that result in the dysregulation of cellular signaling pathways (2011; Jones et al., 2008). To date, large-scale cancer genomics experiments by next-generation DNA sequencing technologies have detected molecular alterations across a wide range of human cancers (Beroukhim et al., 2010; Kan et al., 2010; Wood et al., 2007). A catalogue of genomic abnormalities that drive cancer progress is essential for the development of novel therapeutics. Furthermore,

genomic alterations as biomarkers to guide patient selection for clinical trials are crucial to the success of development of new treatment.

A tumor suppressor gene is a gene that protects cells from becoming cancerous. The most established tumor suppressors include p53, Rb, APC, PTEN, and FBW7, which are frequently inactivated by somatic mutations and genetic deletions in different human malignancies (Li et al., 1997; Su et al., 1993; Welcker and Clurman, 2008). Inactivation of these tumor suppressor genes results in constitutive hyperactivation of various oncogenic signaling pathways, leading to uncontrol cell proliferation and tumorigenecity. For instance, Inactivating mutations in APC gene, which encodes the tumor suppressor adenomatosis polyposis coli (APC), leads to the activation of the WNT pathway and are often found in colorectal cancer cells (Kinzler and Vogelstein, 1997; Weinstein, 2002). Restoration of APC function blocks activation of the WNT signaling pathway through phosphorylation and degradation of β-catenin. PTEN (phosphatase and tensin homolog) is one of the most commonly silenced tumor suppressors in many human cancers, such as glioblastoma, prostate, and breast cancer (Li et al., 1997). Loss of functional PTEN in cancer cells leads to constitutive activation of the PI3K pathway which include the AKT and mTOR kinases.

A wide range of methodologies were adopted for identification of tumor suppressor. Traditional genetic and cellular methodologies allow us to uncover a number of tumor suppressor and their functions. For example, gain-of and loss-of-function analyses for these tumor suppressor genes are necessary to validate

the function of such genes as tumor suppressors during cancer development. Moreover, recent development of technologies for whole-genome sequencing, copy number analysis and expression profiling enables the generation of comprehensive molecular descriptions of tumor suppressor genes, which allow the identification of new oncogenic signaling that are dysregulated by inactivation of these tumor suppressor genes in the malignancy (Berger et al., 2011; Beroukhim et al., 2010; Kan et al., 2010; Stratton et al., 2009).

1.1.2 Aberrant DNA methylation as a cause of tumor suppressor genes silencing in cancer

Epigenetic regulation is a heritable gene expression changes that occurs without alteration in genomic DNA sequence. DNA methylation involves addition of a methyl group to the 5' position of cytosines within CpG dinucleotides, which is mainly mediated by DNA methyltransferases (DNMTs) such as DNMT1, DNMT3a and DNMT3b (Rhee et al., 2002). DNA hypermethylation has been well-established to be a crucial mechanism that results in silencing of tumor suppressor genes (Herman and Baylin, 2003). For instance, the DNA hypermethylation of *CDKN2A* (cyclin-dependent kinase inhibitor 2A) (Herman et al., 1995; Merlo et al., 1995), *hMLH1* (mutL homologue-1), and *BRCA1* (breast-cancer susceptibility gene 1) (Esteller, 2000; Herman and Baylin, 2003) have led to their loss of expression in many solid tumors (Baylin et al., 2000). More recently, epigenetic inactivation of WNT antagonists such as secreted frizzled-related gene family (SFRPs) and

Dickkopf3 (DKK3) activates the Wnt/β-catenin pathway, thereby promoting the growth of cancer cells (Baylin and Ohm, 2006; Suzuki et al., 2004; Yue et al., 2008). Studies have shown that the genome wide profiles of DNA methylation of tumor suppressor genes are specific to the cancer type (Esteller et al., 2001). Thus, genome-wide profiling epigentic alterations of tumor suppressor genes and their related signaling pathways will provide the new understanding the biological processes underlying .

1.2 The role of tumor suppressor PP2A in cancer development

The serine/threonine protein phosphatase type 2A (PP2A) is a trimeric holoenzyme that serves as a critical cellular regulator of cell growth, proliferation, and survival (Westermarck and Hahn, 2008). Increasing evidences indicate that PP2A works as a tumor suppressor in human cancer. However, the molecular mechanisms by which PP2A activity is inactivated in human cancer is largely unknown. In this section, I will discuss the structure and regulation of PP2A complex. More importantly, I will focus on the regulatory mechanisms of PP2A involved in cellular transformation and discuss the current findings in the molecular mechanisms of PP2A disruption in human malignancies.

1.2.1 PP2A structure

PP2A belongs to the phosphoprotein phosphatase (PPP) family of Ser/Thr phophatases and functions as a trimeric holoenzyme consisting of a catalytic subunit

(PP2A C), a scaffolding A-subunit and one of a large array of regulatory B-subunits (Janssens and Goris, 2001). In mammalian cells, PP2A C subunit is constitutively bound to the structural subunit (PP2A A) to form the core of the enzyme. Variable regulatory B subunits (PP2A B) that associate with the core enzyme determine the specificity of its substrates. PP2A catalytic activity is encoded by two distinct Cα and Cβ subunits (Stone et al., 1987). The catalytic C subunit has a highly conserved domain at the C-terminal tail, which determines the interaction of A subunit and recruitment of B subunit (Longin et al., 2007). Two alternative genes, PPP2R1A (Aα) and PPP2R1B (Aβ), encode the two distinct structural subunits, which differ in their ability to interact with the various regulatory B subunits (Groves et al., 1999). The A subunits primarily serve a structural role and maintain the PP2A holoenzyme composition (Ruediger et al., 1999). The regulatory B subunits have been further divided into four distinct families as shown in the Figure 1.1 and each family consists of several members: B (B55 or PR55), B′ (B56 or PR61), B″ (PR48, PR72, and PR130) and B‴ (PR93/ PR110). Each of the B subunit binds to the A subunit mutual exclusively. More than 200 biochemically distinct PP2A complexes were discovered from differential combinations of A, B, and other subunits. The diversity of PP2A heterotrimers suggests that particular regulatory subunits mediate specificphysiological functions through regulating specific substrates in different mammalian tissues (Virshup and Shenolikar, 2009). However, the roles of specific PP2A complexes in the cellular functions remain elusive.

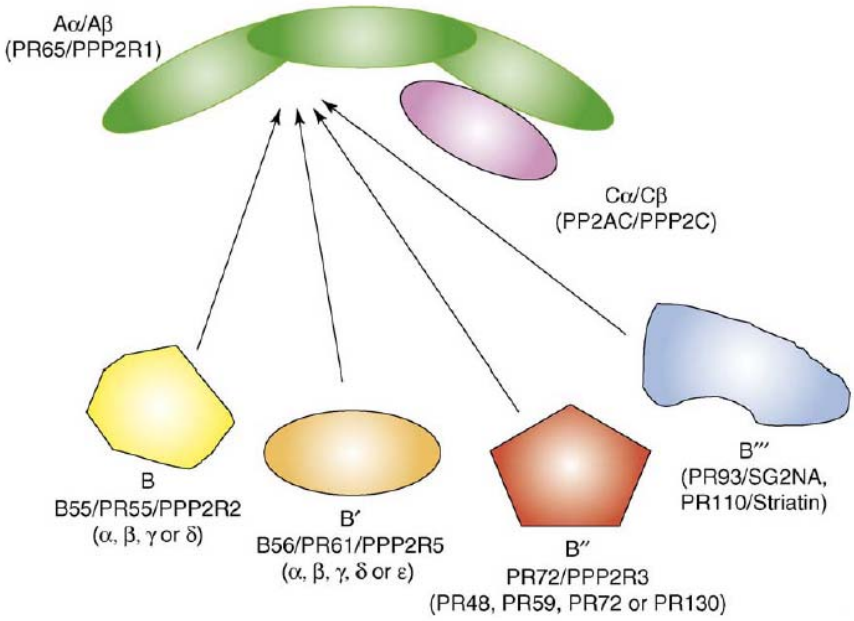


Figure 1.1 The structure of PP2A complex

PP2APP2A is a heterotrimeric complex composed of a structural A subunit, a catalytic C subunit (pink) and one of several B regulatory subunits (yellow, orange, red and blue). B subunits regulate the activity and localization of PP2A complexes. Several forms of each of these subunits exist in humans, and thus many different enzymatic complexes can be formed. (Westermarck and Hahn, 2008)

1.2.2 The regulation of PP2A activity

PP2A has serine/threonine protein phosphatase activity that functions to dephosphorylate various kinases that are involved in many different signaling pathways. Virus infection and somatic mutations can cause the disruption of PP2A complex and loss of functions in cellular process. For example, inactivating mutations of structural A subunit disrupt the ability of scaffolding to form an active PP2A complex with specific regulatory subunits, leading to cellular transformation (Arroyo and Hahn, 2005).

In addition, the activity of PP2A could also be regulated by a number of other

cellular and viral proteins. For instance, the SV40 small T antigen alters PP2A activity by displacing the regulatory B subunit from the holoenzyme complexes (Pallas et al., 1990). Recent studies indicated that phosphorylation and methylation of the C-terminal tail of the catalytic PP2A subunit (PP2A C) play an important role in the regulation of both catalytic activity of PP2A C and recruitment of different B subunits to the PP2A complex. PP2A structural study indicated that PP2A is regulated through the post-translational modification such as methylation by a methylating enzyme, LCMT, and methyl esterase PME-1 (Xing et al., 2008). Generally, the cellular activity of PP2A complex is dependent on the binding partner of its core dimer and post translational modification, resulting in the control of various cellular processes, including cell growth, adhesion, and cytoskeletal dynamics. In particular, recent studies have elucidated roles for PP2A in cell transformation and tumorigenesis (Junttila et al., 2007; Sablina et al., 2007).

1.2.3 PP2A functions in transformation models

PP2A plays an integral role in the regulation of a number of major signaling pathways, including cell proliferation, survival and cell transformation. However, the activity of PP2A in many cellular processes has been an impediment to defining its role as a tumor suppressor. Genetic manipulation of the catalytic C or scaffolding A subunits affects the phosphorylation of hundreds of proteins in many cellular processes, which make it difficult to dissect the functions of PP2A in cellular transformation and other cellular processes.

PP2A was first suggested to act as tumor suppressor based on the okadaic acid as selective inhibitor of PP2A (Suganuma et al., 1988). OA was shown to inhibit PP2A activity and potently promoted tumors in a mouse model of carcinogenesis, which was later demonstrated to be caused by the activation of several oncogenic signaling pathways. The second evidence came from the discovery that PP2A was the target of several tumor-promoting viruses such as simian virus SV40 and polyoma virus (Andrabi et al., 2007; Hahn et al., 2002). Interestingly, the alteration of PP2A by viral proteins leads to the deregulation of similar pathways which were found to be disturbed by okadaic acid. For instance, ST specifically replaced B56γ to disrupt the PP2A complex and inhibit its activity in this ST-dependent transformation model.

Several reports have proposed the underlying molecular mechanisms of cellular transformation driven by altered PP2A function. Recent studies indicate that pyST appears to preferentially activate the MAP kinase pathway while ST stimulates AKT phosphorylation in a PP2A-dependent manner (Andrabi et al., 2007; Chen et al., 2005). By using this transformation model dependent on the tumor-promoting viral antigen, several groups have identified pathways and proteins that are involved in the tumor suppressor functions of PP2A. For example, Myc was previously identified as a direct target of PP2A regulation (Arnold and Sears, 2006). PP2A holoenzyme containng the B56α dephosphorylates Myc at Serine 62 targeting Myc degradation. Conversely, inactivation of PP2A by ablation of B56 or ectopic expression of ST results in Myc stabilization and contributes to cellular

transformation (Arnold et al., 2009; Yeh et al., 2004). Moreover, by using a comprehensive loss-of-function approach, Sablina showed that manipulation of 4 distinct PP2A complexes results in human cell transformation through activation of c-Myc, Wnt, and PI3K oncogenic pathways (Sablina et al., 2010). Taken together, these studies systematically identify the specific PP2A complexes involved in control of cell transformation and define the PP2A-dependent pathways involved in cellular transformation. However, more studies are necessary to provide a more complete view of the molecular mechanisms by which specific PP2A complexes affect these oncogenic pathways in human malignancies.

1.2.4 Mechanisms and cellular consequence of PP2A disruption in human cancer

PP2A complexes regulate a variety of signaling pathways involved in cellular transformation. However, the precise role of PP2A deregulation during tumor progression is not clear and the mechanism by which PP2A dysfunction induces tumorigenesis remains elusive. Furthermore, it is also possible that different set of genetic and/or epigenetic alterations during tumor formation require the loss of different PP2A complexes for the tumor to survive. As such, the role of PP2A as a tumor suppressor is likely to be more diverse than initially suggested and to be largely context-dependent. While the evidence exists implicating that PP2A complexes play important roles in human cell transformation, investigation of a direct relevance to human cancer has been so far limited. The majority of the work

has involved screening tumor samples and cell lines for somatic mutations in PP2A subunit genes. For example, somatic mutations in the PP2A A subunits have been reported in human lung, breast and colon cancers, although at low frequency (Wang et al., 1998). Biochemical studies confirmed that PP2A A subunits mutations disrupt the ability of such mutants to form PP2A complexes and the cancer–associated PP2A A subunits mutants are functionally defective in binding to specific B subunits and in phosphatase activity. For instance, PP2A complex containing B56γ subunit regulates the phosphorylation of AKT and cancer-associated A subunit mutations lead to haploinsufficiency, loss of Aα complexes containing B56 and eventually increased phosphorylation of AKT and tumor formation (Chen et al., 2005). In contrast, cancer-associated PP2A Aβ subunit mutations lead to the complete loss of function of PP2A complexes and increased RalA GTPase phosphorylation (Andrabi et al., 2007; Sablina et al., 2007). These findings suggest that loss or alteration of PP2A activity by cancer-associated mutations is an essential step in tumor development and supports the notion that PP2A acts as a tumor suppressor in human malignancies. Although these observations suggest that cancer-associated PP2A A subunits mutants contribute to human cell transformation, the low frequency of mutation in PP2A subunits limit the wider implication of PP2A as a tumor suppressor in many human cancers.

In addition to the PP2A structural A subunits' somatic mutations, aberrant expression of PP2A subunits are observed in human cancers. For example, PP2A B56γ deletion was found in lung cancer and reactivation of B56γ inhibits cancer cell

growth *in vitro* and *in vivo* (Chen et al., 2004). Another link between PP2A complexes and tumorigenesis is found in the Wnt signaling pathway, which play important role in cancer development. The adnomatous polyposis coli (APC), axin and glycogen synthase 3β form a Wnt regulated signaling complex that mediates the phosphorylation-dependent degradation of β-catenin. PP2A holoenzyme forms a specific complex with APC through its regulatory B56α subunit. Overexpression of this subunit causes proteasomal degradation of β-catenin and thereby inhibits Wnt signaling in human cancer (Morin et al., 1997; Seeling et al., 1999).

In contrast to genetic inactivations of PP2A subunits, overexpression of endogenous PP2A inhibitor proteins, such as SET and CIP2A, might also inhibit PP2A tumor suppressor activity (Junttila et al., 2007; Neviani et al., 2005). Recent work has identified that SET was overepressed in chronic myelogenous leukemia (CML) and its expression was correlated with the oncogenic activity of BCR-ABL kinase. Moreover, the phosphatase activity of the tumor suppressor PP2A is inhibited by the BCR-ABL-induced expression of SET. Specifically, reactivation of PP2A by ablation of SET or by pharmacological inhibition of BCR-ABL led to Myc degradation and also inhibition of other PP2A targets. Consequently, these observations indicate that functional inactivation of PP2A might be essential for BCR-ABL leukemogenesis and also required for blastic transformation. CIP2A (cancerous inhibitor of PP2A) was previously identified as PP2A inhibitor protein by using a proteomic approach. It was shown that CIP2A can stabilize Myc protein by inhibiting the catalytic activity of the PP2A towards Myc S62 phosphorylation. In

contrast, further functional studies indicated that CIP2A is overexpressed in two human malignancies including head and neck squamous cell carcinomas (HNSCCs) and colon cancers. Importantly, ectopic expression of CIP2A can also replace ST to promote cell transformation. Consistent with a role of CIP2A in transformation, knockdown of CIP2A by short interference RNA (siRNA) in squamous cell carcinoma cell lines expression high levels of CIP2A markedly blocks the tumor formation through inhibition of Myc.

In summary, the studies discussed above have established that PP2A is a bona fide tumor suppressor protein. A variety of mechanisms for inhibiting PP2A are present in transformed cells and disruption of PP2A complexes is a common feature in human malignancy. However, as the majority of evidence supporting the role of PP2A as tumor suppressor has been obtained by using viral antigens or chemical inhibitors, the in vivo mechanisms by which PP2A activity is inhibited remains elusive in different cancer types. Moreover, further studies are necessary to identify alterations in PP2A and/or its inhibitor proteins, as these alterations might serve as biomarkers for cancer diagnostic and targeted therapies. Taken together, unraveling the mechanisms of PP2A signaling in human cancer may provide new insights into cancer development and identify novel targets for cancer therapy.

1.3 The mTOR pathway and cancer

The phosphatidylinositide 3-kinase (PI3K) pathway is most commonly activated in a wide range of human cancers in regulating cell growth, proliferation,

and metabolism (Engelman et al., 2006; Wong et al., 2010). Different genetic and epigenetic alterations were reported for the aberrant activation of this pathway. First, activating mutations of PI3K upstream activators such as RAS or receptor tyrosine kinases (RTKs), enhance PI3K activity in many cancer types (She et al., 2008). Secondly, this pathway is also activated by activating mutations of PIK3CA or inactivation of PTEN (Li et al., 1997; Samuels et al., 2004). In this section of review, I will briefly introduce the mTOR pathway and its cellular functions. More importantly, I will highlight the recent progress of the aberrant activations of mTOR signaling in human cancer and the contribution of mTOR to cancer development.

1.3.1 Overview of PI3K/AKT/mTOR signaling pathway

The components of the PI3K pathway include upstream activators of PI3K enzyme (such as RTKs and Ras), PI3K catalytic subunit p110 and regulatory subunit p85, PTEN, downstream effectors (such as PDK1, AKT and mTOR) and transcription factors (such as c-Myc and IKK). Figure 1.2 shows a simplified overview of the PI3K-AKT-mTOR pathway. Briefly, PI3K are activated by cell-surface receptors, such as receptor tyrosine kinases (RTKs), G protein-couple receptors (GPCRs), and RAS. and subsequently catalyzes the conversion of PIP2 to PIP3. Conversely, phosphatase and tensin homolog (PTEN) is a tumor suppressor that antagonizes PI3K activity by converting PIP_3 to PIP_2. PIP_3 initiates the activation of AKT to activate multiple downstream effectors, including mTOR, forkhead transcription factor (FOXO), glycogen synthnase kinase-3 (GSK3), and

BCL2-associated agonist of cell death, which regulate multiple cellular processes, such as metabolism, proliferation, and survival. Among these downstream effectors, mTOR signaling is extensively studied as it is hyperactivated in human malignancies and drugs targeting its activity are now in clinical use.

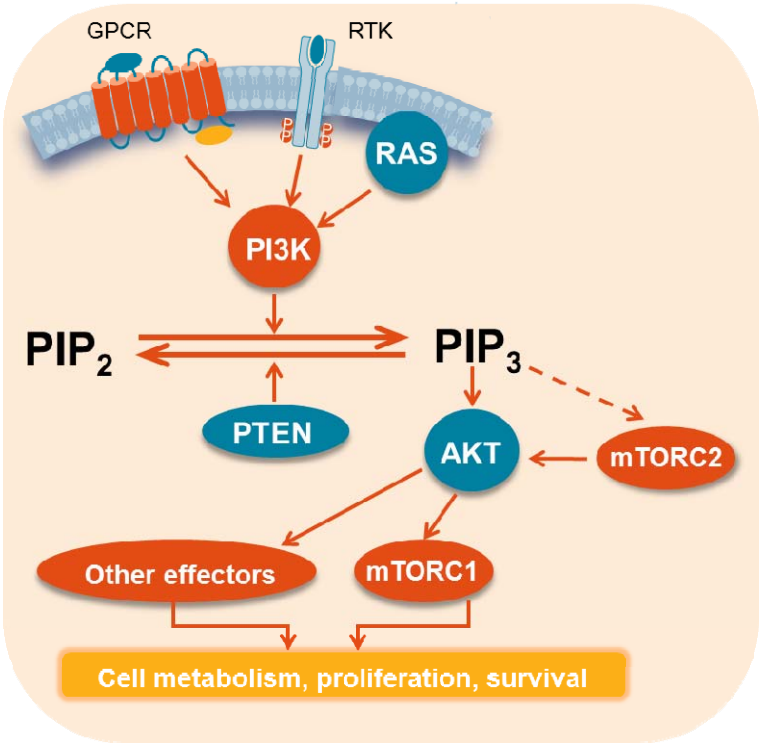


Figure 1.2 A simplified overview of the PI3K-AKT-mTOR pathway

GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; mTOR mammalian target of rapamycin; mTORC1, mTOR complex 1; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate; PIP2, phosphatidylinositol-3,4,5-diphosphate; PTEN, phosphatase and tensin homolog.

1.3.2 mTOR signaling components and cellular function

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that has been identified as a critical downstream effector of PI3K/AKT signaling pathway. mTOR signaling regulates a series of cellular functions including cell growth by

controlling mRNA translation, ribosome biogenesis, autophagy, and metabolism through its downstream effectors such as S6 kinase (S6K) and 4EBP1 (Guertin and Sabatini, 2005; Sarbassov et al., 2005). mTOR exists in two distinct intracellular complexes called mTOR complex1(mTORC1) and mTOR complex2 (mTORC2). As shown in the figure 1.3, Both mTORC1 and mTORC2 complexes comprise of the mTOR catalytic subunit with different regulatory-associated proteins of mTOR, such as Raptor (mTORC1) and Rictor (mTORC2) (Guertin and Sabatini, 2007; Hay and Sonenberg, 2004; Sancak et al., 2007). The mTORC1 complex is sensitive to rapamycin and its analogs; conversely, mTORC2 is a rapamycin-insensitive complex (Sarbassov et al., 2004).

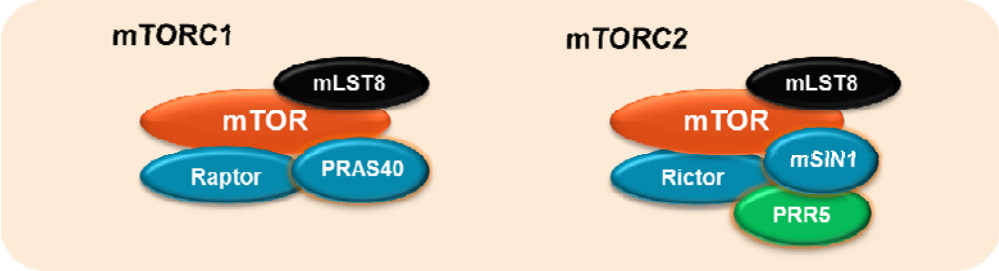


Figure 1.3 The mTORC1 and mTORC2 complexes

As shown in the Figure 1.4, mTORC1 is activated by AKT through a pathway that involves the tuberous sclerosis complex (TSC1-TSC2) as well as the small G protein Ras homolog enriched in brain (Rheb). Activated mTORC1 phosphorylates translational regulator S6K1 and the eukaryotic translation initiation factor 4E (eIF-4E) binding proteins (4E-BP1 and 4E-BP2), which are the only extensively described mTORC1 substrates (Sarbassov et al., 2006). Activated S6K phosphorylates the ribosomal protein S6, controlling the rate of translation and promoting cell growth; while activated 4E-BP1 release the initiation factor elF4E,

allowing the initiation of translation (Gingras et al., 2004). S6K also works as a key upstream effector of mTORC1, activated S6K inhibits the insulin receptor substrate 1(IRS1), suppressing IRS1-mediated activation of the PI3K pathway, which leads to a negative feedback that downregulates PI3K signaling (Sarbassov et al., 2005). In contrast, mTORC2 activates AKT (at serine 473) and SGK1 and is part of the PI3K pathway. mTORC2-induced phosphorylation of S473 in the C-terminal hydrophobic motif is necessary for the full activation of AKT (Alessi et al., 1996). Furthermore, ablation of the mTORC2 components mTOR or rictor, but not raptor by loss-of-function RNAi experiments leads to a complete loss of AKT S473 phosphorylation in a variety of mammalian cells (Sarbassov et al., 2006; Sarbassov et al., 2005). Taken together, these findings suggest that mTORC2 directly activates AKT to regulate cell survival.

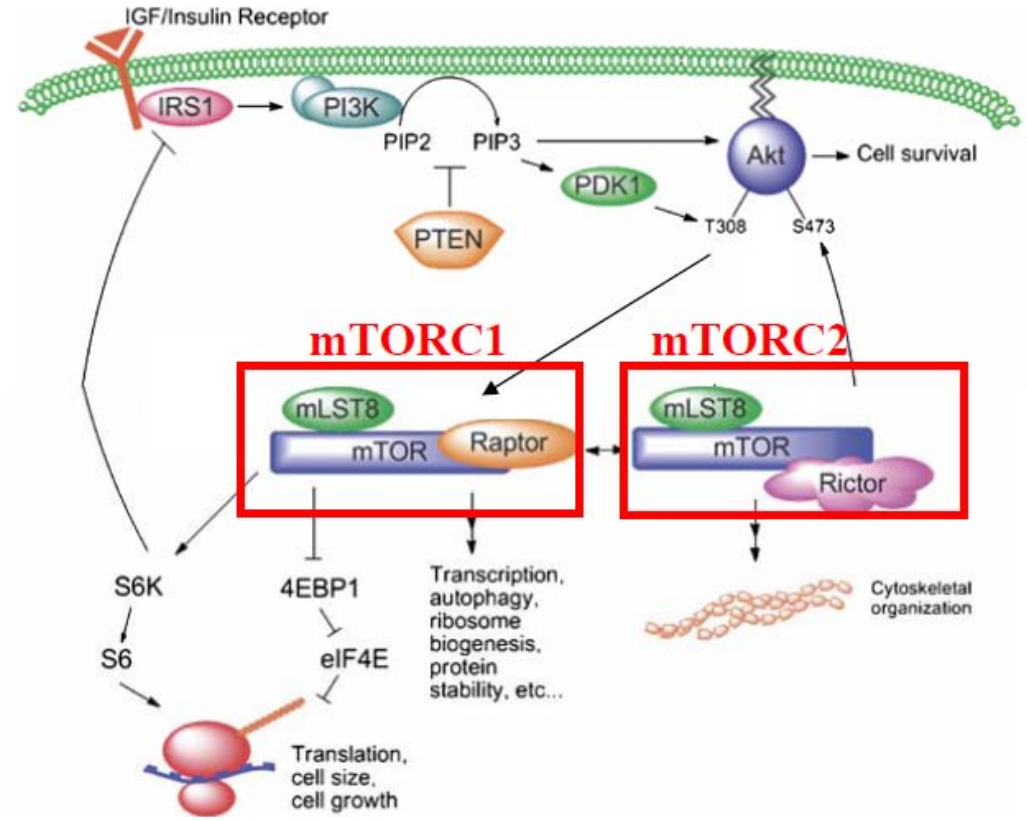


Figure 1.4 The mTOR signaling pathway.

The functional mTOR signaling complex exists in two forms: mTORC1 and mTORC2. Growth factor stimulation signals through PI3K to activate AKT leading to mTORC1 activation. mTORC2 may also activate AKT via phosphorylation of Ser473. Two major substrates of mTORC1 are 4E-BP1 and S6K, whose phosphorylation promotes the translation of key cell cycle regulators and transcription factors.

mTOR plays a key role in the regulation of cell proliferation, angiogenesis, autophagy, and cell metabolism. For instance, mTOR regulates cell proliferation by promoting the translation of cyclinD1 and c-Myc (Nelsen et al., 2003). Furthermore, activated mTOR may also play an additional role in cell cycle progression through the regulation on the production of p21, the regulatory protein that stops the cell cycles in cell proliferation (Beuvink et al., 2005). Thus, inhibition of mTOR leads to

cell cycle arrest or growth inhibition. In addition, mTOR activation also promotes angiogenesis through control the protein synthesis of hypoxia inducible factor 1α/β (HIF1α/β), which are subunits of a master transcription factor that mediates the expression of genes that produce angiogenic factors (Pouyssegur et al., 2006; Semenza, 2003). Since mTOR activity is also controlled by the cellular environment, it is also a critical inhibitor of autophagy (Crazzolara et al., 2009). Recently bioenergetics research has shown that mTOR plays a key role in regulating cell metabolism (Wullschleger et al., 2006).

1.3.3 Deregulation of mTOR hyperactivity in cancer

Although genetic alterations of mTOR have not been reported in human cancers, numorous reports indicate that deregulation of upstream pathway effectors can lead to hyperactivation of the mTOR signaling. For example, loss or inactivating mutations of tumor suppressors such as PTEN and TSC1/2 promote PI3K-dependent activation of mTOR (Sabatini, 2006).

In addition to the alterations of the upstream effectors, genetic amplification of its downstream effectors such as elF4E, S6K1 and 4E-BP1, result in enhanced mTOR activation, which contributes to its hyperactivity in human cancer. For instance, amplification of elF4E has been linked to a wide range of human cancer, which also mediates oncogenic transformation (Kentsis et al., 2004). Moreover, the overexpression of S6K and downregulation of 4E-BP1 were reported in breast, ovarian, and other cancers (Dowling et al., 2009). Notably, a recent report showed

that active S6K directly phosphorylates the tumor suppressor PDCD4 (programmed cell death protein 4), targeting it for degradation and leading to cancer (Dorrello et al., 2006). However, S6K is also involved in the negative feedback inhibition of AKT, its contribution towards tumorigenesis might be limited. Thus, identification of new mTOR substrates will be important to understand the mechanisms by how mTOR signaling deregulated in human cancer.

Recent studies showed that silencing of targets of mTORC1 with tumor suppressive function are also involved in the aberrant of mTOR signaling during cancer development. For example, Grb10 (growth factor receptor-bound protein 10) was recently identified as mTORC1 substrate, was found to be frequently downregulated in various cancers. Experimental results showed that mTORC1 phosphorylates and promotes Grb10 stabilization, resulting in feedback inhibition of the PI3K and ERK-MAPK pathway (Hsu et al., 2011; Yu et al., 2011). Taken together, these findings link aberrant activation mTOR signaling to genetic alterations in cancer. As a result, mTOR has emerged as an important target for anti-cancer therapy.

In summary, this part of the review collectively shows the various components and cellular functions of mTOR pathway. In cancer cells, genetic alterations of key components in the PI3K pathway result in a constitutive activation of mTOR signaling, which make these components attractive targets for cancer therapy, such as small molecules inhibitors targeting RTKs, PI3K, and mTOR. However, increasing evidence show that acquired drug resistance is emerging as a significant

issue that impedes the clinical success of the PI3K-targeted therapy. Therefore, identification of biomarkers to predict clinical response in cancer therapy will be pivotal for understanding the resistance mechanisms in PI3K-targeted therapy.

1.4 Targeting PI3K pathway in cancer therapy

The PI3K/AKT/mTOR pathway is the most commonly deregulated pathway in human cancer (Vivanco and Sawyers, 2002). PI3K is activated by upstream activators such as oncogenic receptor tyrosine kinase or RAS. Many components in the PI3K-AKT pathway are protein kinases, such as RTKs, PI3Ks, AKT, and mTOR; the oncogenic activations of these kinases make them as ideal anti-cancer drug targets. Many of PI3K pathway inhibitors are currently in clinical trials and show great promise for the treatment of PI3K pathway-addicted tumors (Engelman, 2009). As shown in Figure 1.5, inhibitors that target key components in the PI3K/AKT/mTOR pathway, including RTKs (such as EGFR, HER2 and VEGFR), PI3Ks (such as P110 catalytic subunits and p85 regulatory subunit), AKT and mTOR, have very encouraging clinical results in PI3K pathway-targeted therapy (Liu et al., 2009). However, the efficacy of these targeted agents may have limited clinical success due to the complex cross-talks between various pathways and feedback loops, resulting in the acquired drug resistance during targeted therapy. Thus, in this review I will describe different strategies for targeting PI3K pathway in cancer therapy. In addition, we will discuss the molecular mechanisms of acquired drug resistance in PI3K-targeted therapy which dramatically leads to limitations of

clinical application.

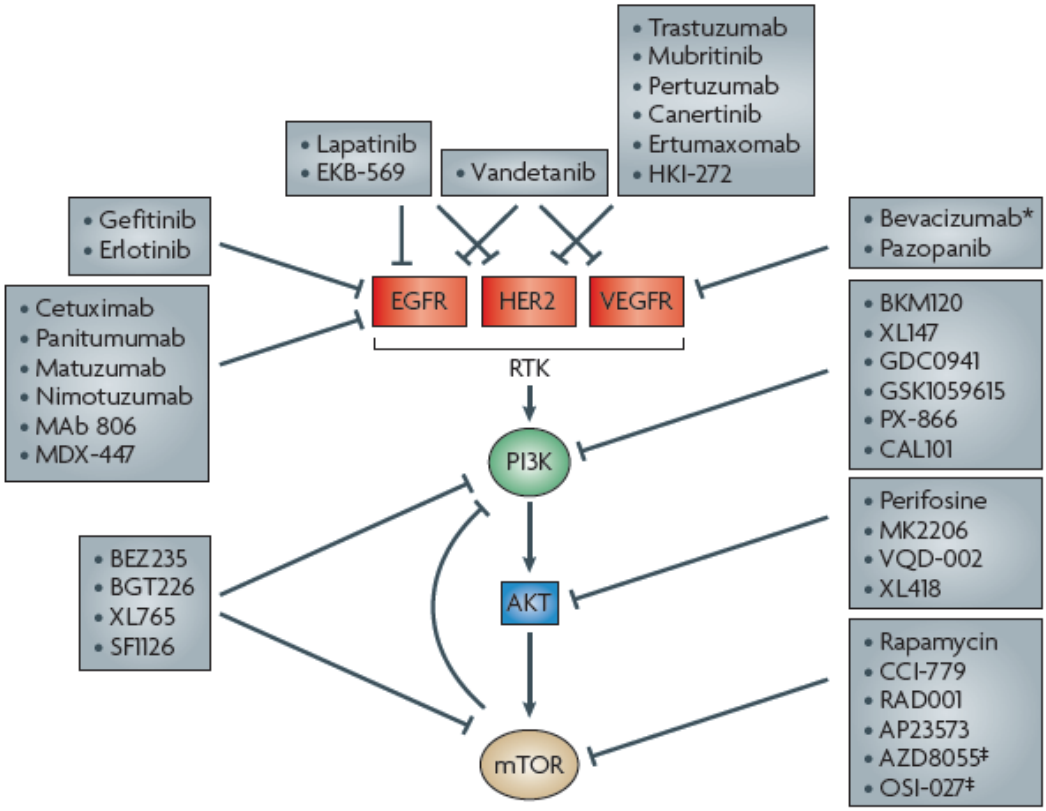


Figure 1.5 Targeting the PI3K pathway in cancer.

Inhibitors in clinical development that target the PI3K or related pathways are shown. EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HER2, human epidermal growth factor receptor 2 (also known as ERBB2); VEGFR, vascular endothelial growth factor receptor (Liu et al., 2009).

1.4.1 Targeting the RTK-PI3K-AKT in cancer therapies

Aberrent alterations of RTKs and RAS through somatic mutations and gene amplification invariably activate the PI3K pathway. Small molecule RTK inhibitors such as EGFR inhibitors (erlotinib and gefitinib) and HER2 inhibitor (lapatinib) are the most established targeted therapies in clinical use, which specifically targets cancer cell with activating mutations of RTKs. These RTK inhibitors achieve their

anti-tumor effects, at least in part, by disabling the PI3K/AKT pathway through blocking the signaling from RTKs to PI3Ks (Junttila et al., 2009).

Although targeting RTKs has become an important therapeutic approach for a wide range of human cancers, the effectiveness of such drugs is restricted by the development of drug resistance through different mechanisms in human cancer (Engelman and Janne, 2008). Multiple mechanisms leading to drug resistance to RTK inhibitors have been well-documented, including secondary target mutations, gene amplification, and compensatory activation of pro-survival signaling pathways. Intriguingly, these mechanisms can occur to the same drug in the same disease. For example, secondary mutation in the kinase domain of EGFR (T790M) was found in 50% of gefitinib-resistant patients, this mutation increases the affinity for ATP as WT EGFR and weakens the affinity for EGFR kinase inhibitors (Li et al., 2007; Yun et al., 2007). On the other hand, amplification of the MET gene which encodes for the receptor tyrosine kinase for hepatocyte growth factor has also been made resistant to the EGFR inhibitor (Engelman et al., 2007; Kobayashi et al., 2005; Pao et al., 2005). Furthermore, a recent report suggested that multiple RTKs are coactivated to drive and maintain downstream signaling to PI3K pathway, thereby limiting the efficacy of therapies targeting single RTKs in brain tumor (Stommel et al., 2007). Interestingly, all these potential resistance mechanisms lead to constitutive activation of the downstream PI3K or MEK signaling pathway (Engelman et al., 2007). Conversely, activation of the downstream signaling components (such as AKT and MEK) or inactivations of PTEN also result in resistance to RTK inhibitors. Indeed, it

has been recently shown that loss of PTEN or activation of PIK3CA in breast cancers with amplifications of ERBB2 can confer resistance to trastuzumab treatment (ERBB2 inhibitor) by setting a high threshold of Akt activation (Berns et al., 2007). Thus, these findings have led to clinical trials using newly designed RTK-targeted therapies that can overcome these resistance mechanisms.

Frequent somatic mutations in the PIK3CA gene which encodes class IA PI3K catalytic subunit p110α can lead to constitutive activation of PI3K signaling and oncogenic transformation, making PIK3CA a druggable target in cancer therapy (Liu et al., 2009). Many PI3K inhibitors have been developed in clinical setting, including pan-PI3K inhibitors, and PI3K catalytic isoform-specific inhibitors. Pan-PI3K inhibitors target all class IA PI3K catalytic subunits, such as p110α, β, γ and δ isoforms. Currently, several pan-PI3K inhibitors, including GDC0941, PX-886, BKM120, and XL147 (Ihle et al., 2005; Liu et al., 2009; Markman et al., 2010), have entered clinical trials with low toxicity and high anti-tumor activity. In addition, isoform-specific inhibitors that selectively inhibit different p110 catalytic subunits are under investigation in preclinical studies. For example, CAL-101, a PI3Kδ specific inhibitor, is in clinical trials for hematological malignancies (Herman et al., 2010).

Despite the promise of PI3K-targeted therapies, an emerging clinical obstacle is the acquired resistance to PI3K inhibitors, leading to treatment failure. A recent study showed that certain mutations in PIK3CA would confer resistance to the PI3K inhibitors (Zunder et al., 2008). Unlike the RTK inhibitor resistant mutations, these

PIK3CA mutations did not reside in the classic gatekeeper residues. In addition to resistant mutations in PIK3CA, PI3K inhibitors might not effectively down-regulate AKT activity in cancers with AKT activating mutations or gene amplification (Carpten et al., 2007). Moreover, a feedback upregulation of receptor tyrosine kinase ERBB3 expression and activity can also lead to acquired resistance to PI3K inhibitors in patients with HER2-overexpression breast cancer (Chakrabarty et al., 2011). Given the feedback loops attenuate anti-tumor effect of PI3K inhibitor, a combined inhibition of PI3K and RTKs may be a potentially useful strategy to bypass or prevent drug resistance in the clinic.

AKT is the most crucial proximal downstream component of the PI3K pathway. Both ATP-competitive inhibitors and allosteric inhibitors have been developed, including perifosine, MK-2206 and GSK690693 (Hirai et al., 2010; Rhodes et al., 2008). These AKT inhibitors could be particularly effective in treating cancers harboring mutations and amplifications of AKT or increased pathway activity. However, the anti-tumor effects of AKT inhibitors may be attenuated by the relief of negative feedback loops. For example, one report shows that AKT inhibitors promote RTK expression and activity by activating FOXO and inhibiting mTORC1, thereby leading to persistent activation of PI3K-AKT and MEK-ERK pathway (Chandarlapaty et al., 2011). On the other hand, Vasudevan and his colleages reported that AKT is often not required for proliferation of cancer cells with activated PI3K pathway (Vasudevan et al., 2009). In this case, the prevalence and dependence of PDK1 in PI3K signaling might substantially affect the clinical

outcome of AKT inhibitors. Of notice, PDK1 inhibitors are currently developed as anti-cancer agents that have been shown to be effective in vitro and in vivo in cancer (Maurer et al., 2009; Peifer and Alessi, 2008). However, the molecular mechanisms in which pathway is inhibited by this class of kinase inhibitors remain elusive.

1.4.2 Utility of mTOR inhibitors in human cancers and resistance mechanisms

Since hyperactivation of the mTOR signaling occurs in diverse human malignancies, inhibition of this kinase has became an attractive therapeutic strategy to treat cancer (Guertin and Sabatini, 2007, 2009). Rapamycin and its analogues, such as RAD001 and CCI-779 (referred to as rapalogs), suppress mTOR activity through binding to the FKBP12 binding protein, which in turn interact and inhibit mTORC1-mediated phosphorylation of the substrate S6K and 4EBP1 (Alessi et al., 2009; Guertin and Sabatini, 2009; Meric-Bernstam and Gonzalez-Angulo, 2009). Although preclinical studies have shown that members of this class of mTOR inhibitors have activity against a number of cancer types, rapalogs have achieved limited success as anti-cancer agents. Mechanistically, these rapalogs only inhibit the mTORC1 complex, partially reducing phosphorylation of 4EBP1 in most cell context (Janes et al., 2010). In addition, inhibition of mTORC1 releases the feedback loops that inhibit PI3K-AKT and MAPK-ERK signaling pathways. In many cancer cells, this leads to elevated AKT and ERK activity and promotes cell survival (Carracedo et al., 2008b; Sarbassov et al., 2006; Sarbassov et al., 2005). These

findings provide a strong rationale for dual targeting of mTORC1 and its feedback activities. Consistent with this idea, several dual inhibitors of PI3K and mTOR activity have been developed including PI-103 and NVP-BEZ235 (Chiarini et al., 2010; Cho et al., 2010; Fan et al., 2006; Maira et al., 2008). These agents have shown greater anti-cancer efficacy compared with those rapalogs. However, the clinical therapeutic efficacy and tolerability of such dual PI3K-mTOR inhibitors remain to be established. The second-generation ATP-competitive mTOR inhibitors, such as PP242 and Torin1, and thus target kinase domain of mTOR, repressing both mTORC1 and mTORC2 activity (Dancey, 2010; Janes et al., 2010). Because mTORC2 leads to an increase in activation of AKT (Sarbassov et al., 2005), these ATP-competitive inhibitors of mTOR maybe superior over rapalogs in cancer therapy by blocking the feedback activation of AKT and suppressing 4EBP1 phosphorylation followed by its downstream effectors. In addition to activating AKT and ERK signaling, rapalogs treatment also results in the stimulation of multiple other pro-survival feedback loops, including activation of PIM kinase, altered expression of eIE4E and 4EBP1, and gene amplification of Myc (Dilling et al., 2002; Graff et al., 2008; Hammerman et al., 2005; Ilic et al., 2011). Accordingly, pharmacological or genetic inhibition of these pathways has promoted sensitization to mTOR-targeted therapy.

Sensitivity to mTOR inhibitors is thought to be related to dysregulation of critical upstream components of PI3K/mTOR pathway. Results from preclinical models suggest that genetic alterations and aberrant gene expression resulting in

pathway activation correlate with sensitivity to mTOR inhibition. For example, HER2 amplification in breast cancer; upregulation of HIF1α; PIK3CA activating mutations elevated levels of phosphorylated AKT in many human cancers; and Cyclin D1 overexpression have been identified in preclinical models as putative predictors of rapalogs response (Brachmann et al., 2009; Hsieh et al., 2010; Majumder et al., 2004; Serra et al., 2008; Thomas et al., 2006). However, the use of PTEN and PIK3CA alterations and AKT phosphorylation for predicting rapalogs sensitivity has not been fully validated. This outcome may be due to the complexity of the PI3K/AKT/mTOR signaling network and the existence of feedback loops and cross-talk between oncogenic signaling pathways involved in malignant transformation, which might determine sensitivity of mTOR inhibitors. Thus, it is critical to understand the genetic alterations in human tumors with acquired resistance to mTOR inhibitors for insights into additional resistance mechanisms, and these findings will provide new effective mTOR-targeted therapies for cancer patients.

1.4.3 Potential clinical implications for targeting PI3K pathway

Although targeting the PI3K-AKT-mTOR pathway remains a promising anticancer strategy, PI3K-targeted inhibitors in clinical development are likely to be limited due to the emergence of acquired drug resistance. Acquired resistance can reduce the effectiveness of an anti-cancer or render the anti-cancer completely ineffective against human malignancies. Identification of reliable biomarkers to

predict the treatment response to PI3K-targeted therapies is indeed urgent for the successful clinical trials. Therefore, a broader and more systematic interrogation of genetic and epigenetic alterations is required to facilitate patient selection and to monitor treatment efficacy in clinical use of PI3K inhitiors. In this section, we have discussed the most recently identified molecular mechanisms that mediated acquired resistance to current PI3K-targeted therapies. The knowledge obtained from these studies will help us to design better therapies that prevent and overcome resistance to treatment in cancer patients. Furthermore, these insights have prompted the development of new PI3K inhibitors.

While dual PI3K/mTOR inhibitors and ATP-competitive mTOR inhibitors may prove more potent as single agents than rapalogs, their greatest efficacy may lie in simultaneous targeting of more than one pathway. Mounting evidences indicate that anti-cancer efficacy and response duration with inhibitors of a single kinase are frequently limited by parallel signaling, bypass pathway activation, or feedback activation. Thus, the outcome of ongoing clinical studies of RTKs, PI3K, AKT and mTOR inhibitors as well as further testing of the combination of these agents will be a key for the development of targeted therapeutics in a wide range of human cancer.

1.5 Researh objectives

Genome-wide mapping of genetic and epigenetic alterations has great clinical potential for cancer therapies as they give rise to virtually key pathways as potential targets. The preliminary purpose of this thesis is to discover the novel tumor

suppressor genes silenced by DNA methylation in colorectal cancer. In order to achieve this objective, a comprehensive genomic analyses, including gene expression, H3K4me3 and DNA methylation, were used in colorectal cancer.

PP2A complex works as known tumor suppressor gene that regulates variety of kinases and oncogenic signaling pathways. However, the oncogenic lesions leading to PP2A inactivation in colorectal cancer has not been documented. By using intergrative genetic and epigenetic analysis as described above, we have revealed that PPP2R2B which encodes a specific PP2A B regulatory subunit was silenced by DNA methylation in most of the colorectal cancer. Given the clinical evidence pointing to a wide occurrence of PP2A tumor suppressor inactivation has not been previously studied in human malignancies, the present study is mainly focused on investigating the silencing mechanism and the potential roles of PPP2R2B in CRC. Furthermore, this study also aims to investigate the impact of PP2A in oncogenic signaling and therapeutic responses to targeted therapy, especially in the known oncogenic PI3K/AKT/mTOR signaling pathway. More specifically, the following studies were conducted:

1. To identify novel tumor suppressor genes silenced by DNA methylation;

2. To determine the silencing mechanism of PPP2R2B, one of CMS genes identified by above approach, in colorectal cancer cells;

3. To identify the potential tumor suppressor function of PPP2R2B *in vivo* and *in vitro*;

4. To investigate the potential substrates of PPP2R2B-associated PP2A complexes;

5. To investigate the resistant mechanism of mTOR inhibitor rapamycin in colorectal cancer and the role of PP2A-PPP2R2B in mTOR inhibitor senstivity;

6. To determine a novel oncogenic pathway PDK1-Myc, which is closely related to the loss of PPP2R2B tumor suppressor function, in colorectal cancer and the role of this pathway in rapamycin sensitivity in cancer therapy;

7. To examine the combination therapy by using PDK1 inhibitor and mTOR inhibitor in colorectal cancer.

The present study identifies a new tumor suppressor pathway which is inactivated in CRC through inactivation of PP2A subunit. Further elucidation of the pathway revealed a dysregulated pathway mediated by PDK1, which leads to rapamycin resistance in CRC. Thus this project provided a potential therapeutic strategy in treating rapamycin-resistant colorectal cancer.

2 Chapter II: Materials and Methods

2.1 Cell lines and cell culture

2.1.1 Colorectal cancer cell lines

The human colorectal cancer cell lines HCT116, DLD1, RKO, SW480, HT29, HT15, SW403 were purchased from American Type Culture Collection (Manassas, VA) and cultured with Dulbecco's modified Eagle's media (DMEM), supplemented with 10% FBS (Invitrogen). HCT116 cells with genetic disruption of DNMT1 and DNMT3B (HCT116 DKO) were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) (Rhee et al., 2002) and cultured in the same medium as colorectal cancer cell lines.

2.1.2 Other cell lines

The immortalized human embryo kidney epithelial cells (HEK-TERV) were kind gifts from Dr. W.C. Hahn at Dana-Farber Cancer Institute (Chen et al., 2004). 293T and the immortalized human mammary epithelial cells (MCF-10A) were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in culture as recommended by ATCC. The human hepatocellular carcinoma cell lines (HCC) Hep3B, HepG2, hUH7 and human osteosarcoma cells U2OS were were routinely maintained in DMEM media with 10% fetal bovine serum (FBS; Invitrogen). Ovarian cancer cells OVCAR3, OVCAR5, SK-OV-3 and Human renal cell carcinoma (RCC) cell lines 786-O and Caki-2 were used and culture with RPMI 1640 medium supplemented with 2mM L-glutamine, 10μg/ml insulin and 5% fetal

bovine serum. All cells were cultured at 37°C in a 5% CO2 humidified incubator.

2.2 Patient tumor and normal samples

Colorectal primary tumors and matched adjacent non-malignant colon tissues were obtained from Singapore Tissue Network in accordance with the local ethics committee. All tissue samples were harvested after obtaining written informed consent from patients. Normal colon DNA samples were purchased from Stratagene (Stratagene, La Jolla, CA).

2.3 Drugs and chemicals

5-aza-2′-deoxycytidine(5-AzaC) and Doxycycline were purchased from Sigma-Adrich (St.Louis, MO) and prepared in sterile distill water. Rapamycin and PI-103 were purchased from Alexis (San Diego, CA). PDK1 inhibitor BX912 and the PIK3CA inhibitor PIK90 were obtained from Axon Medchem (Groningen, Netherlands). All kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO).

2.4 RNA analysis

2.4.1 Total RNA isolation

Total RNA was isolated by homogenizing cells in TRIzol (Invitrogen) followed by subsequent purification using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. In brief, cells were lysed with 1ml TRIzol and 200µl chloroform. The supernant layer containing RNA was separated by centrifuge and

mixed with equal volume of 70% ethnol. The mixtures were purified with RNeasy Mini kit and RNAs were eluted into 30µl RNase-free water. The concentration and qualitity of the total RNA were determined by a NanoDrop (ND-1000) (NanoDrop Technologies, Delaware, USA). Extracted RNAs were stored at -80°C.

2.4.2 Reverse transcriptase (RT)

The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (ABI) according to the manufacturer's instructions. Briefly, 1.0µg of total RNA was reverse transcribed into complementary DNA (cDNA) in a 20µl reaction mixture consisting of RT Buffer, dNTPs, random primers and Multiscribe reverse transcriptase, incubated at 25°C for 10 minutes and then at 37°C for 2 hours. The products of RT were further processed by gel-based semi-quantitative RT-PCR or quantitative real-time PCR.

2.4.3 Polymerase chain reaction (PCR)

2.4.3.1 Gel-based semi-quantitative RT-PCR

RT-PCR was carried out in a thermocycler machine (DNA Engine DYAD, Bio-Rad) in a final volume of 12.5µl reaction containing 1.0µl of synthesized cDNA (100ng/reaction) as template, 0.6µM of each forward or reverse primers (Table2.1), and 9.5µl of Taq-Gold PCR master mix (ABI). The conditions of PCR program were dependent on the specificity of PCR primers. *GAPDH* or *ACTIN* were used as internal control. The PCR products were visualized by 2% DNA agarose gel

electrophoresis.

Table 2.1 Oligonucleotide primers for RT-PCR

Table 2.1 Oligonucleotide primers for RT-PCR

Name	Direction	sequence
SFRP1	Forward	5' TCGGCCGCGAGTACGACTA 3'
	Reverse	5' TCTTGTAGCCCACGTTGTGG 3'
CDH2	Forward	5' CCCTGCTTCAGGCGTCTGTA 3'
	Reverse	5' CATGCCATCTTCATCCACCT 3'
THY1	Forward	5' GACCCGTGAGACAAAGAAGC 3'
	Reverse	5' GCCCTCACACTTGACCAGTT 3'
RASD1	Forward	5' GAACTGCTATCGCATGGTCA 3'
	Reverse	5' AACGTCTCCTGTGAGGATGG 3'
HAND1	Forward	5' AGTGGGCTGCGCGTCTCATTTTC 3'
	Reverse	5' AAGGGTTCGTGGAGCATGGG 3'
DDIT4L	Forward	5' ATGGTTGCAACTGGCAGTTTG 3'
	Reverse	5' AGGACCTTTGAGCAACCAAG 3'
ADRB2	Forward	5' AACGGCAGCGCCTTCTTGCT 3'
	Reverse	5' GGCCCATGACCAGATCAGCA 3'
PPP1R3C	Forward	5' ATGAGCTGCACCAGAATGATCC 3'
	Reverse	5' TTGGTTGTGTGAGCACTTCC 3'
PPP2R2B	Forward	5' ATGGAGGAGGACATTGATACC 3'
	Reverse	5' ACATTGTATTCACCCCTACG 3'
SOX17	Forward	5' ATACGCCAGTGACGACCAGAGC 3'
	Reverse	5' TAGCCCACACCATGAAAGCG 3'
GAPDH	Forward	5' ATGGGAAGGTGAAGGTCGG 3'
	Reverse	5' AAGACGCCAGTGGACTCCACGA 3'
ACTIN	Forward	5' GTGGGGCGCCCCAGGCACCA 3'
	Reverse	5' CTCCTTAATGTCACGCACGATTTC 3'

2.4.3.2 Quantitative real time PCR

Quantitative real-time PCR of *PPP2R2B* or Myc was performed on a PRISM 7900 Sequence Detection System (Applied Biosystems) using TaqMan probe of *PPP2R2B* (Assay ID: Hs00270227_m1, Applied Biosystems) and Myc (Assay ID: Hs00905030_m1, Applied Biosystems) with Taqman fast universal PCR Master mix

kit (Applied Biosystems). Three independent experiments were performed and samples were normalized to the levels of *GAPDH* mRNA (Taqman®*GAPDH* control reagent of human). Data was analyzed by using ABI SDS 2.2 software (Applied Biosystems).

2.4.4 Microarray analysis

Total RNA was subjected to reverse transcription to synthesize the first-strand cDNA and amplify the second-strand cDNA synthesis by using the Illumina® TotalPrep™ RNA Amplification kit (Ambion, USA) according to the manufacturer's protocols. The resultant amplified RNAs were hybridized on the Sentrix® HumanRef-8_V2 Expression BeadChip which covered approximately 24,000 RefSeq transcripts for whole-genotyping expression assay (Illumina, USA). The beadChip was washed and scanned with Illumina BeadStation according to the Illumina protocols. Microarray scanned images were imported to Illumina® GenomeStudio for data quality control and the raw data was analyzed with GeneSpring GX 11.0.2 (Agilent Technologies) (software settings: Lower cutoff for 'Present' call: 0.95; Upper cutoff for 'Absent' call: 0.6; Threshold raw signal to: 10; Normalization algorithm: Quantile; Baseline Transformation: median of all samples), the gene expression level data file were transformed to log2 values and quantile normalized. Significant genes were selected based on a minimum 3 fold change and FDR < 0.1%. Unsupervised hierarchical clustering analysis for the significant genes was performed using Cluster and visualized using TreeView.

2.4.5 Gene ontology analysis and clinical relevance analysis

The colon cancer methylation silencing genes (CMS) were imported to Ingenuity Pathway Analysis (www.ingenuity.com) for gene ontology (GO) analysis and analyzed results showed the GO biological functions and potential signaling pathways involved. To get the information of gene expression pattern on published databases, we examined the *PPP2R2B* expression level in Oncomine database (www.Oncomine.org). Oncomine provides web-based cancer/normal microarray database (Rhodes et al., 2004a, b). The expression level of *PPP2R2B* in each cancer vs normal database was exported as box plots.

2.5 Chromatin immunoprecipitation (ChIP)-sequencing assay

2.5.1 Chromatin immunoprecipitation

ChIP assays with HCT116 and DKO cells were carried out as following: Cells were cross-linked with formaldehyde (Sigma-Aldrich) directly to culture medium to a final concentration of 1.0% for 10 min at room temperature. Formaldehyde was inactivated by addition of 200mM glycine. After wash with ice-cold PBS, cells were harvested and lysed in ChIP lysis buffer followed by sonication to shear the chromatin-DNA. Chromatin extracts containing DNA fragment of average size of 500 bp were immunoprecipitated using anti-H3K4me3 (Upstate) and rabbit IgG (Santa Cruz). After a series of washing with wash buffers, the DNA-protein complex

was eluted in ChIP elution buffer and the DNA was extracted by Phenol:Chloroform:IAA (Ambion). The purified ChIP-DNA was dissolved in TE buffer and ready for ChIP-seq experiments.

2.5.2 ChIP-seq

Purified immunoprecipitated DNA was prepared for sequencing according to a modified version of the solexa genomic DNA protocol. Briefly, fragmented DNA was end paired and subjected to 15 cycle of linker-mediated (LM)-PCR using Oligos purchased from Illumina. Amplified fragments between 200 and 300 bp were isolated by agarose gel electrophoresis and purified. The fragment DNA size was confirmed by Agilent DNA chip analysis. Ten nanograms of linker-ligated DNA were applied to the flow-cell using the Solexa Cluster Station fluidics device. Samples were then subjected to 36 bases of sequencing according to Illumina's standard protocols. The data generated from Solexa Genome Analyzer instrument will undergoes ChIP-seq pipeline, manage by the Research Computing group.

2.6 DNA analysis

2.6.1 Purification of genomic DNA

Genomic DNA was extracted from cell pellets according to the recommended protocol from the QIAamp® DNA Mini Kit (QIAGEN). The cell pellets were resupsended in 200µl of 1X PBS and 20µl of proteinase K. The samples were further mixed and washed in the spin column with buffers provided after which the DNA

was eluted in sterile distilled water.

2.6.2 DNA bisulfite treatment

Bisulfite modification of DNA was performed by using the EZ DNA methylation-Gold kit (ZYMO Research) according the manufacture's instructions. Briefly, 1.0µg of gemomic DNA in 20µl distilled water was added to 130µl of conversion treatment reagent and EZ DNA PCR program was carried out at 98°C for 10mins, followed by 8 cycles of 53°C for 30mins, 53°C for 6mins and 37°C for 30mins. After EZ DNA program, 600µl M-Binding buffer was added into Zymo-Spin IC Column and place the column into provided collection tube and the PCR samples were loaded into the column and centrifuged at full speed >10000×g for 30secs. The columns were washed with 200µM Desulphonation buffer and 200µl M-Wash buffer to the column. Bisufited DNAs were eluted with 10µl M-Elution buffer and stored at -20°C for DNA methylation analysis.

2.6.3 DNA promoter and CpG island prediction

DNA promoter was identified and downloaded from genotype database in Genaltas (http://genatlas.medecine.univ-paris5.fr) and the CpG island of promoter was predicted by using CpG island searcher: http://www.uscnorris.com/cpgislands/cpg.cgi or Methyl Primer Express v1.0.(Invitrogen).

2.6.4 DNA methylation analysis

The CpG island DNA methylation status was determined methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS). MSP and BGS primers were designed by using Methyl primer express v1.0 (Invitrigen) and synthesized. MSP and BGS were performed in a 12.5µl reaction mixture consisting of 0.6µM of each primers targeting specific DNA promoters (Table2.2), 0.2mM dNTP, 1.5mM $MgCl_2$, 1×PCR buffer, 0.5unit Gold-Taq (Applied Biosystems) and 0.5µl of bisulfited template DNA. PCR program was carried out at 95^{o}C for 10 min, followed by 40 cycles of 94^{o}C for 30 s, 60^{o}C for 30 s, 72^{o}C for 30 s and a final cycle of 72^{o}C for 10 min (MSP) or 30 min (BGS). The PCR products were analyzed on 2.0% agarose gels. The MSP results of selected samples were confirmed by bisulfite genomic sequencing. BGS PCR products are ligated with TA cloning vector (PCR2.1–TOPO, invitrogen) and transformed with TOP-10 competent cells (Invitrogen). 10 clones are chosen for sequencing and the methylation ratio of each CpG site is tabulated.

Table 2.2 Oligonucleotide primers for Methylation-specific PCR and Bisulfite genomic sequencing.

Name	Direction	sequence
SFRP1 M	Forward	5' TGTAGTTTTCGGAGTTAGTGTCGCGC 3'
	Reverse	5' CCTACGATCGAAAACGACGCGAACG 3'
SFRP1 U	Forward	5' GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT 3'
	Reverse	5' CTCAACCTACAATCAAAAACAACACAAACA 3'
CDH2 M	Forward	5' AGTACGTACGATTCGGAGC 3'
	Reverse	5' GCGAAAAATACAACGACG 3'
CDH2 U	Forward	5' TGTAGTATGTATGATTTGGAGT 3'
	Reverse	5' ACCACAAAAAATACAACAACA 3'
HAND1 M	Forward	5' CGTTTTTAGGTTTCGGTGC 3'
	Reverse	5' CGACGAATTTATTCCCGACT 3'
HAND1 U	Forward	5' GTGTTTTTAGGTTTTGGTGT 3'
	Reverse	5' TCAACAAATTTATTCCCAACT 3'
DDIT4L M	Forward	5' GTCGTGTATTCGGAATTGC 3'
	Reverse	5' ATAACCTCCGAAACAACGAA 3'
DDIT4L U	Forward	5' GAGTTGTGTATTTGGAATTGT 3'
	Reverse	5' ACATAACCTCCAAAACAACAAA 3'
THY1 M	Forward	5' TATTTTTATATTAATGCGGGATCGT 3'
	Reverse	5' CGATTACTACACCCAACTCGAA 3'
THY1 U	Forward	5' TTATTTTTATATTAATGTGGGATTGT 3'
	Reverse	5' TCCAATTACTACACCCAACTCAAA 3'
RASD1 M	Forward	5' AGAGGGATAGGGGTATCGTC 3
	Reverse	5' CCTCCCAACTCGATACGT 3'
RASD1 U	Forward	5' GTAGAGGGATAGGGGTATTGTT 3'
	Reverse	5' ACCCTCCCAACTCAATACAT 3'
PPP1R3C M	Forward	5' CGGATATATCGGGTAATGAAC 3'
	Reverse	5' CGTACCGCATACAACGAAC 3'
PPP1R3C U	Forward	5' ATGTGGATATATTGGGTAATGAAT 3'
	Reverse	5' CATACCACATACAACAAACACC 3'
ADRB2 M	Forward	5' GGTAGAACGTATTGCGAAGC 3'
	Reverse	5' GAAACTCTACGAACCGCG 3'
ADRB2 U	Forward	5' GGGTAGAATGTATTGTGAAGT 3'
	Reverse	5' CAAAACTCTACAAACCACA 3'
PPP2R2B M	Forward	5' AGTAGTAGTTGCGAGTGCGC 3'
	Reverse	5' GAACAACCGCGACAAAATAAT 3'
PPP2R2B U	Forward	5' AGTAGTAGTAGTTGTGAGTGTGT 3'
	Reverse	5' AAACAACCACAACAAAATAATACC 3'
SOX17 M	Forward	5' ACGTGGGATTCGGATTAC 3'
	Reverse	5' CGCCCGTATTCTAACCTATC 3
SOX17 U	Forward	5' GATGTGGGATTTGGATTAT 3'
	Reverse	5' CCACCCATATTCTAACCTATC 3'

PPP2R2B BGS	Forward	5' ATTATTGTTGTTGGGAAAGA 3'
	Reverse	5' CAAAATAATACCTTTCTAAACCC 3'

M: methylated primer; U: unmethylated primer; BGS: bisulfite genomic sequencing primer.

2.7 Plasmid Construction

2.7.1 Mamalian expression plasmid construction

The human full-length *HAND1*, *RASD1* and *THY1* cDNA were isolated by RT-PCR using 100ng total RNA from normal colon tissue (BD bioscience) with gene cloning primers with indicated restriction enzyme sites (Table 2.3). The PCR products were cloned into mammalian expression vector pcDNA4/Myc/hisB (Figure 2.1).

Table 2.3 Oligonucleotide primers for expression vector construction

HAND1	Forward	5' GGTACCACCATGAACCTCGTGGGCAGCTA 3'
	Reverse	5' GAATTCCACTGGTTTAACTCCAGCG 3'
THY1	Forward	5' GGTACCACCATGAACCTGGCCATCAGC 3'
	Reverse	5' GAATTCCACAGGGACATGAAATCCGTG 3'
RASD1	Forward	5' GGTACCACCATGAAACTGGCCGCGATGA 3'
	Reverse	5' GAATTCCAGCTGATGACGCAGCG 3'

The human Full-length *PPP2R2B* coding region was isolated by RT-PCR using 100 ng total RNA from normal colon tissue and the following primers with indicated restriction enzyme site at 5′ and 3′ respectively: Forward primer with KpnI 5′-GGTACCACCatggaggaggacattgatacc-3′ and Reverse primer with XhoI 5′-CTCGAGCAgttaaccttgtcctgga-3′. The PCR product was cloned into pcDNA4/myc-hisB (Figure 2.1) between Kpn I and Xho I sites for overexpression studies or pcDNA4/TO/myc-hisB for inducible system (Figure 2.2). The human full-length PDK1 was amplified by RT-PCR using 100ng total RNA from HEK293 cells and the following primers with EcoRI restriction enzyme site: 5'-tcGAATTCgccaggaccaccagccagc-3' and 5'- GAGAATTCctgcacagcggcgtccgg-3'. The PCR product was cloning into pHA.CE vector (Figure 2.3) between EcoRI and sequenced. To generate *PPP2R2B* retroviral expression vector, myc-tagged *PPP2R2B* fragment was subcloned into pMN-IRES-GFP vector (Figure 2.4) (a kind gift from Dr. Linda Penn's Laboratory) between BamH I and Xho I sites.

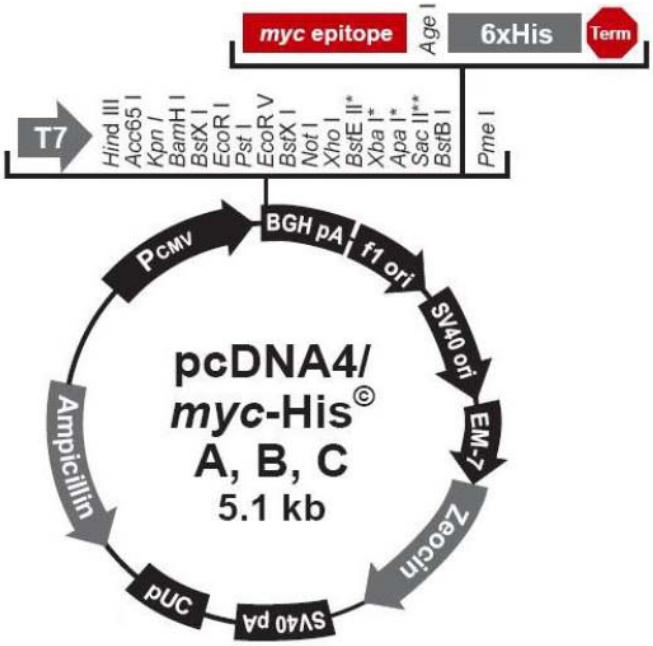


Figure 2.1 Map of mammalian expression vector pcDNA4/myc-his

Type B was used in this thesis. Gene of interest was inserted into multicloning sites(MCS) and fused with myc-tagged.

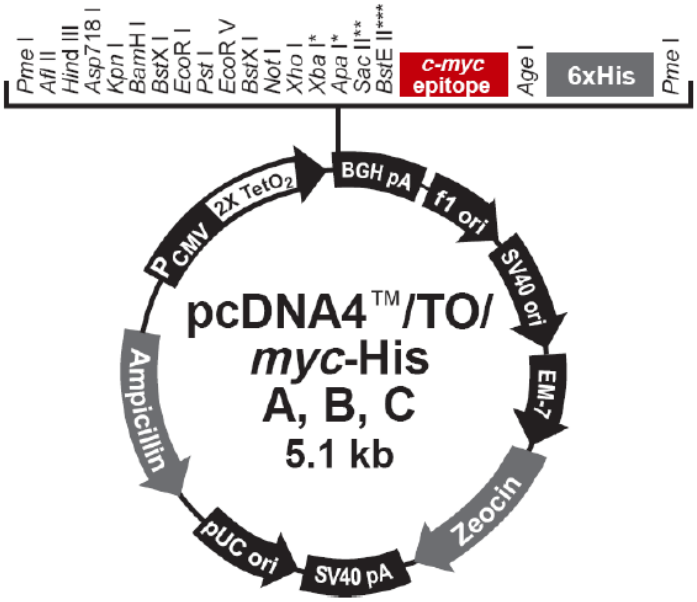


Figure 2.2 Map of mammalian expression vector pcDNA4/TO/myc-his in T-REx™ system

Type B was used in this thesis. Gene of interest was inserted into multicloning sites(MCS) and fused with myc-tagged.

pHACE

Epitope tag : C-terminal HA tag (YPYDVPDYA)

Cloning site : EcoRI (GAA-TTC)

Sequence :

HindIII-KpnI-BamHI-XhoI-GCCACC-ATG-GAA TTC-TAT CCT TAC GAC GTG-

Kozak M **EcoRI** Y P Y D V

CCT GAC TAC GCC-TAA-BamHI-EcoRV-BstXI-NotI-XhoI-XbaI-ApaI

P D Y A STOP

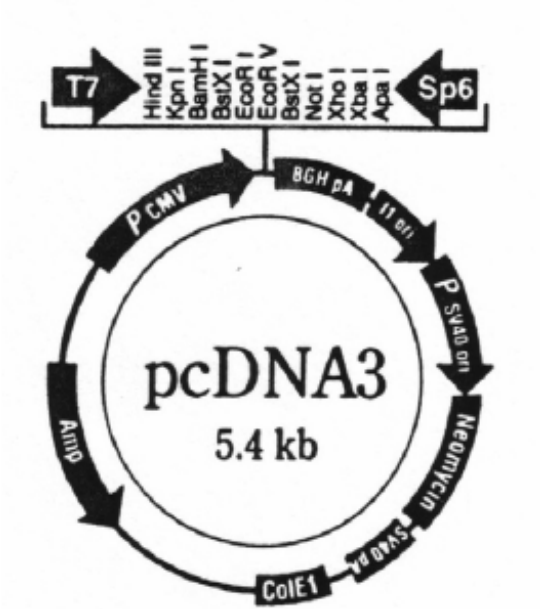


Figure 2.3 Map of mammalian expression vector pHACE with C-terminal HA tag

This vector was constructed from pcDNA3 as vector backbone. PDK1 fragment was inserted into EcoRI cloning site without start codon ATG and stop codon. The mammalian antibiotic selection marker is Neomycin.

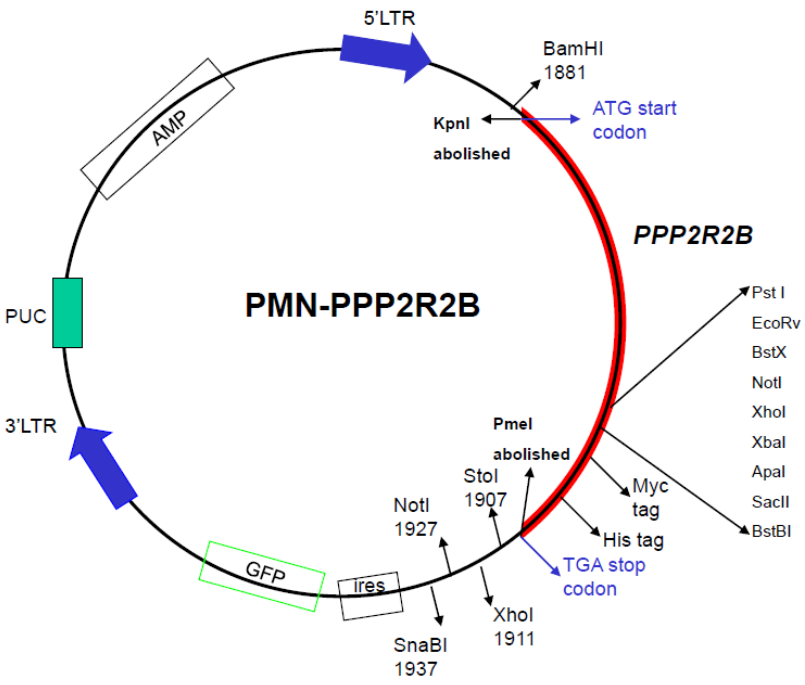


Figure 2.4 Schematic view of retroviral expression vector with PPP2R2B gene.

2.7.2 Construction of pSIREN-RetroQ-ZsGreen1 Vector targeting *PPP2R2B*

PPP2R2B siRNA sequence containing 19bp nucleotides were input into online shRNA sequence designer provided by Clontech (http://bioinfo.clontech.com/rnaidesigner). shRNA forward and reverse primers with MluI site are list in Table 2.4

Table 2.4 PPP2R2B shRNA primer sequence

PPP2R2B-shRNA-F	5'gatccGAATGCAGCTTACTTTCTTTTCAAGAGAAAGAAAGTAAG CTGCATTCTTTTTTACGCGTg---- 3'
PPP2R2B-shRNA-R	5'attcACGCGTAAAAAAGAATGCAGCTTACTTTCTTTCTCTTGAAAA GAAAGTAAGCTGCATTCg---- 3'

shRNA targeting PPP2R2B was constructed into pSIREN-RetroQ-ZsGreen1 vector (Figure 2.5) according to the manufacturer's protocol. Briefly, forward and reverse primers were annealed to double-strand oligonucleotide and ligated with pSIREN-RetroQ-Zsgreen1 vector. shRNA plasmid DNA was selected by single restriction enzyme digestion at MluI.

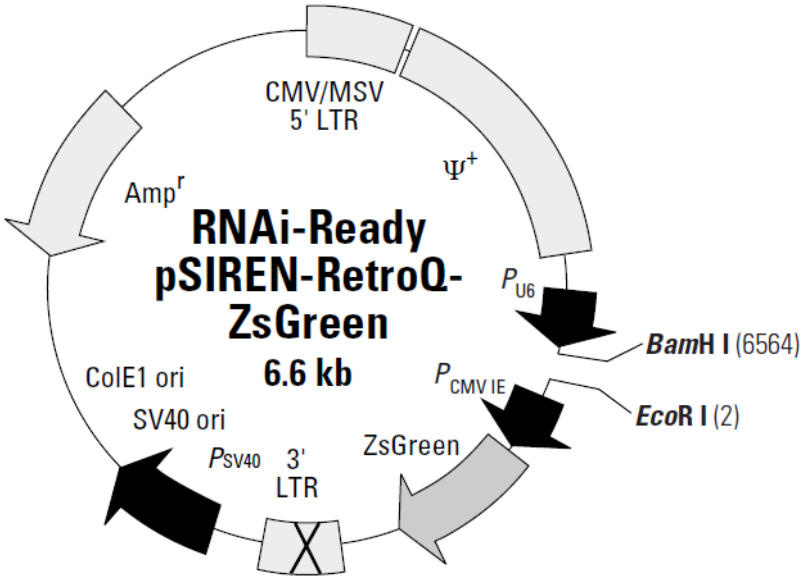


Figure 2.5 Map of RNAi-Ready pSIREN-RetroQ-ZsGreen vector.

Annealed double-strand oligonuleotide shRNA was inserted between BamH I and EcoRI sites. The selection marker of this vector is ZsGreen.

2.8 Generation of stable cell lines

2.8.1 Tet-on inducible Cell lines

Tet-on inducible cell lines were constructed with T-REx™ System (Invitrogen) according to the protocol. Tetracycline regulation in the T-REx™ System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest. To generate the *PPP2R2B* Tet-on inducible cell lines, colon cancer cells DLD1 or HCT116 were transfected with pcDNA6.0/TR (Figure 2.6), followed by selection with Blasticidin (10μg/ml) according to the protocols of T-Rex system kit (Invitrogen), and then transfected with the pcDNA4/TO/PPP2R2B-myc or empty control vector and selected for Zeocin (100μg/ml). The generated clones were screened for doxycycline inducible expression of *PPP2R2B* by using immunoblotting analysis with anti-myc antibody. For doxycycline treatment, inducible cells were treated with doxycycline to a final concentration of 1μg/ml. positively identified clones were expanded and maintained under conditions of 10μg/ml blasticidin and 100μg/ml zeocin.

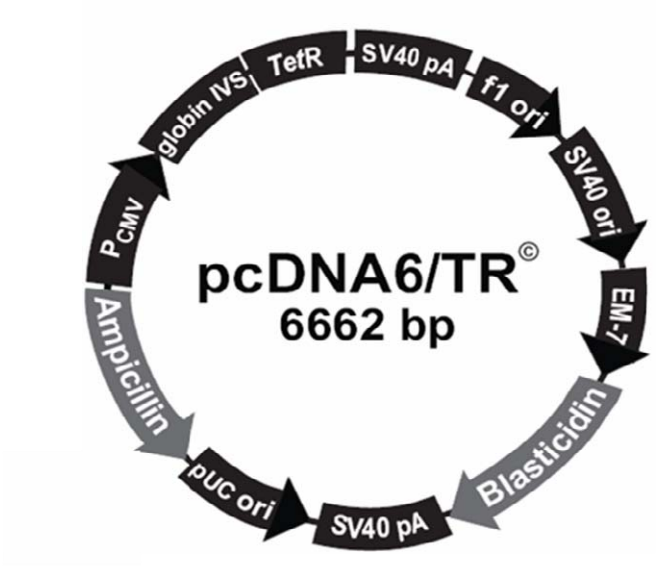


Figure 2.6 Map of pcDNA6/TR vector of Tet-on inducible system.

It expresses the E. coli Tn10-encoded tetracycline repressor which will bind to the Tet operator in pcDNA4/TO/myc-hisB. The mammalian antibiotic selection marker is blasticidin.

2.8.2 Stable cell lines construction

To make *PPP2R2B* retroviral stable expression cell lines, Plat A retroviral packaging cells (Cell Biolab, San Diego, CA) were transfected with pMN-*PPP2R2B* or pMN empty vector by using Lipofectamine 2000 (Invitrogen). After 48 hours of posttransfection, the retroviral particles containing PPP2R2B were infected targeting cells HT29 or SW480 with polybrene (8μg/ml). Stable retroviral cell lines were amplified and selected by sorting with GFP for further analysis.

2.9 Flow cytometry analysis

Cell cycle analysis was done by DNA content quantification. Briefly, cells were seeded into 6-well plate and treated with indicated drugs for different time points. The cells were harvested by trypsin and fixed with 70% ethanol for 1 hr at 4oC. The fixed cells were washed twice with cold PBS, treated with 100μg/ul of RNaseA for 5 min and stained with 50μg/ml of propidium iodide (PI) for 30 min under light protection. The stained cells were analyzed by FACScalibur (BD Bioscience) and quantified by using CellQuest software (BD bioscience).

For BrdU incorporation assay, cells were treated as above and incubated with BrdU reagent for 5 hours before harvesting. Cells were fixed and stained with the BrdU antibody and 7-AAD according to the manufacture's protocol (BD Bioscience). Stained cells were analyzed by FACScalibur (BD Bioscience) and quantified by

using CellQuest software (BD Bioscience).

2.10 Cell viability/proliferation assay

Cell viability was measured using CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, WI) according to manufacturer's instructions. This assay is a homogeneous method of determing the number of viable cells in culture based on quantification of the ATP present, which signals the presence of metabolically active cells. In brief, 1000 cells/well were seeded in 96-well white flat-bottom plate (Costar) with 100µl medium. After drug treatment for the indicated days, cells were lysed by 50µl of CellTiter-Glo® reagent and the luminescence signals were measured by using Microlumat Plus LB96V system (Berthold Technologies).

2.11 Cell Senescence-associated β-galactosidase staining assays

Cell senescence assay was performed according to the protocol of Senescence Detection kit (BioVision, Mountain view, CA). Senescence cells display increase of cell size, senescence-associated expression of β-galactosidase (SA-β-Gal) activity and altered patterns of gene expression. This assay hisochemically measures the SA-β-Gal activity in cultured cells to detect the senescence cells. In detail, DLD1-*PPP2R2B* inducible cells were seed in 6 well and treated with 1.0µg Doxycycline at indicated days. Cells were washed once with PBS and fixed with Fixative Solution for 15 minutes at room temperature. Fixed cells were washed twice

with PBS and stained with 0.5ml Staining Solution Mix (470µlof staining solution, 5µlof staining supplement and 25µl of 20mg/ml X-gal in DMF) for overnight at 37^{o}C. Stained cells were photographed under a microscope for development of blue color.

2.12 Colony Formation Assay in monolayer and soft agar

Monolayer colony formation assays were performed to evaluate cancer cell growth in vitro, DLD1 and HCT116 cells were plated at 3.0×10^{5} per well using 6-well plates, and transfected with either pcDNA4-*PPP2R2B* or backbone pcDNA4 (2.0µg) using Fugen 6 (Roche) according to the manufacturer's protocol. The cells were replated in triplicates and cultured for 10–15 days in complete DMEM medium containing Zeocin (100µg/ml) and stained with gentian violet after methanol fixation. For DLD1 and HCT116 inducible cells foci formation assay, 1×10^{4} cells were seeded on 6-well plate with or without treatment with Dox or rapamcycin. Cells were grown for 2 weeks and the surviving colonies were stained with gentian violet after methanol fixation, and visible colonies (>50 cells) were counted. Soft agar colony formation assay was used to measure the ability of anchorage-independent growth in vitro. For anchorage-independent growth of inducible cells, cells were suspended in complete DMEM containing 0.3% agar with or without doxycycline and layered on complete DMEM containing 0.6% agar in 6-well plate. After 3-4 weeks, colonies were stained with iodonitrotetrozolium chloride (Sigma) for overnight. Colonies from randomly-selected image areas of three replicate wells were enumerated and

three independent experiments were performed.

2.13 RNA interference

2.13.1 siRNA transient transfection

Specific siRNA oligos targeting Myc, p70S6K, PPP2R1A, AKT1, PIK3CA, PDK1, mTOR, raptor and rictor mRNAs were described previously (Cappellen et al., 2007; Heinonen et al., 2008; Sablina et al., 2007; Sarbassov et al., 2005; Vasudevan et al., 2009) (Table2.5). PIK3CB siRNA was purchased from Santa Cruz Biotech. The SMARTpool® siRNA targeting *PPP2R2B* and the non-targeting control were purchased from Dharmacon (Lafayett, CO). A separate *PPP2R2B* siRNA targeting the following sequence: 5'- GCUUACUUUCUUCUGUCUA-3' was obtained from Sigma-Proligo. Cells were transfected with 100nM final concentration of siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions.

Table 2.5 List of siRNA sequence for the functional study

Gene ID	Symbol	Sequence	Ref.
NM_002467	Myc	GGTCAGAGTCTGGATCACC	(Cappellen et al., 2007)
J02902	PPP2R1A	GACAACAGCACCTTGCAGAGT	(Sablina et al., 2007)
NM_003161	P70S6K	GGACATGGCAGGAGTGTTT	(Heinonen et al., 2008)
NM_006218	PIK3CA	AATGAAAGCTCACTCTGGA	(Vasudevan et al., 2009)
NM_005163	AKT1	CGCGTGACCATGAACGAGT	(Vasudevan et al., 2009)
NM_002613	PDK1-1	GCAGCAACATAGAGCAGTACA	(Vasudevan et al., 2009)
NM_002613	PDK1-2	CAAAGTTCTGAAAGGTGAAAT	(Vasudevan et al., 2009)

2.13.2 Stable RNA interference system

shRNA vector PLKO.1 targeting human mTOR, Raptor, Rictor, PDK1, scramble shRNA plasmid, psPAX2 packaging plasmid and pMD2.G envelope plasmid were purchased from Addgene (Cambridge, MA). To generate PPP2R2B shRNA stable cells, ShRNA vector pSIREN-RetroQ-ZsGreen1 targeting human PPP2R2B was transfected into PlatA packaging cell using Lipofectamine 2000 (Invitrogen). At 48 hr posttransfection, viral supernatants were passed through a 0.45μm nitrocellulose filter and were used to infect HEK-TERV cells with polybrene (8μg/ml). Stable retroviral cell lines were selected by sorting with GFP for further analysis. To generate mTOR, Raptor, Rictor and PDK1 shRNA stable cells, the human embryonic kidney 293T cells were used for production of lentivius. PLKO.1 shRNA vectors targeting mTOR, Raptor, Rictor and PDK1 were transfected into 293T cells along with psPAX2 and pMD2.G by using lipofectamine2000. Procedure for infection is the same as retroviral infection. After infection with these shRNA lentivirus, the target cells were selected with 1.0μg/ml puromycin (Sigma) for 7 days and pooled for experiments.

2.14 Western blot analysis

Cells were harvested and washed twice with cold PBS. Cells were lysed with Radioimmunoprecipitation assay (RIPA) lysis Buffer (50mM Tris-HCl, 1mM EDTA, 150mM sodium chloride, 1.0% NP40, 0.5% sodium deoxycholate and complete protein inhibitor cocktail (Roche)). Cells were lysed on ice before being centrifuged

at 13,200 rpm for 15 min at 4°C. Protein concentrations in the supernant were determined by the Bradford Protein Assay Kit (BIO-RAD, Hercules, CA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a mini-gel apparatus (Mini-Protean II, BioRad Laboratories, Hercules, CA). The percentage of SDS-polyacrylamide gel was adjusted according to the protein molecular weight. After protein separation by SDS-PAGE, proteins were transferred onto a PVDF immunobilon membrane (Millipore, Billerica, MA), and the nitrocellulose membrane was blocked in 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T). After blocking, the immunobilon membrane was blotted with specific primary antibodies followed by HRP-labelled secondary antibodies (GE healthcare, UK). Primary antibodies used in this thesis for western blot were: Myc, p-Myc(T58/S62), p70-S6K, p-p70S6K(T421/S424), p-p70S6K(T389), p-p70S6K(S371), p-AKT (S473), p-AKT (T308), p-PDK1(S241), S6, p-S6(S235/236), p-MEK1/2(S217/221), p-ERK1/2(T202/Y204), p-p38MAPK(T180/Y182), PP2A A Subunit(81G5), p110α and p110β (Cell Signaling Technology, Danvers, MA). β-catenin and PDK1 (BD Biosciences), and anti-PP2A B subunit (B55) (Upstate Biotechnology); α-HA(SC-805) and β-Actin (Santa Cruz Biotech) and Myc (9E10) (Sigma-Aldrich). ECL anti-rabbit (NA934V, GE Healthcare, UK) and ECL anti-mouse (NA931V, GE Healthcare, UK) horseradish peroxidise-linked whole antibodies were as secondary antibodies. Chemiluminescence was detected by ECL Plus Western Blotting Detection Reagent (GE Heathcare, UK) and developed with X-ray film (Kodak,

USA).

2.15 Immunoprecipitation

For immunoprecipitation analysis, 293T cells were transiently transfected with HA-PDK1 and PPP2R2B-Myc by using Fugen HD (Roche). After 48 hr of posttranfection, the cells were lysed with Lysis Buffer (50mM Tris-HCl, 150mM sodium chloride, 1.0% NP40, 0.5% sodium deoxycholate and complete protein inhibitor cocktail(Roche)). The cell lysates were precleared by incubating with the protein A/G agarose for 3 hr under the rocking condition at 4°C . Precleared supernatant collected were then incubated with protein G/A agrarose and 2-5µg of pull-down antibodies such as anti HA-tag (SC-805, Santa, Cruz) or Myc-tag (9E10, Roche) and normal IgG Mouse/Rabbit (depending on the species of pull down antibody used) on the rotator overnight at 4°C. The unbound complexes were removed through three times washing with ice-cold buffers suggested in the protocol. The supernatant were removed after the last wash. Agarose beads which bound with the proteins were stained with SDS-PAGE loading dye mixed with DTT reducing reagent before resolving the precipitated complexes using SDS-PAGE gel electrophoresis and western blotting. To study direct interaction between B55β-Myc and endogenous PDK1, DLD1-*PPP2R2B* cells were treated with Dox for 24 hr and cells were lysed and immunoprecipitated with anti-PDK1 and anti-Myc-tag.

2.16 Protein phosphatase activity assay

PPP2R2B-associated PP2A phosphatase activity was measured by using the PP2A Immunoprecipitation Phosphatase Assay Kit (Millipore). In detail, DLD1-PPP2R2B or DLD1 control cells were suspended in lysis buffer (50mM Tris-HCl, pH 7.4, 7.5% Glycerol, 1mM EDTA, 150mM NaCl, 0.5% NP-40, 1mM Na3VO4, Complete Protease Inhibitor), cleared from debris by centrifugation, incubated with c-Myc (9E10, Roche) followed by incubation with protein A agarose(Roche) for overnight at 4^{o}C. The beads were washed with TBS 3 times and followed by one wash with Ser/Thr Assay buffer. 60µl dissolved phosphopetide (K-R-pT-I-R-R) and 20µl Ser/Thr Assay buffer were added to the beads and incubated by shaking for 10 minutes at 30^{o}C. After briefly centrifuged, 25µl supernant was added to 96-well microtiter plate and mixed with 100µl of malachite Green Phosphate Detection Solution. Fluorescence was measured using a fluorescence microplate reader (Sunrise, Tecan) at 650nm wave.

2.17 Immunofluorescence Analysis

The cells were seeded on the glass slides in 12 well plates. After treatment with Dox for 48 hrs, cells were fixed with 3.7% paraformaldehyde in PBS and permeablized with 0.2% Triton-X100. Cells were sequentially incubated with primary antibodies (anti-Myc or anti-p-PDK1(S241) and Alexa Fluor 488 or Alexa Fluor 546-conjugated secondary antibodies (Invitrogen) for 1 hour each and 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining for 15 mins. They were

then mounted in Fluorsave (CalBiochem) mounting medium. The stained cells were examined by Zeiss LSM510 confocal microscopy.

2.18 Mouse Xenografts and Drug Treatment

The female athymice BALB/c nude mice (5-8 week-old) were housed in the Biological Resource Centre. Mice were implanted subcutaneously in both sides of flank with 3×10^6 DLD1-PPP2R2B or control DLD1 cells, respectively. When tumors reached ~70 mm^3, the mice were divided two groups (8 mice per group) and the doxycycline was administrated by oral gavages at 100mg/kg daily. Tumor progress was monitored by whole body weights and tumor size measurements every other day. For combination treatment with rapamycin and doxycycline, the same dose of doxycycline was given every other day and rapamycin was administered by intraperitoneal injection at 4mg/kg at Day 3, 5, 7 and 11. Tumor diameters were measured every 3-4 days with caliper and tumor volumes were calculated per data time point. Statistical analysis was carried out using independent t-test, P value <0.05 was considered significant. All animal work was performed in accordance with animal experiments guidelines of Singapore.

2.19 Statistical analysis

All values from in vitro assays are expressed as mean ± SD or SEM of at least three independent experiments, unless otherwise states. P values were calculated with the two-tailed Student's t test. A p-value<0.05 is considered statistically significant.

3 Chapter III: Integrative genomic and epigenomic analysis reveals silenced tumor suppressors in colorectal cancer

3.1 Introduction

Genetic and epigenetic alterations have been associated with tumor formation and progression. Recent evidence indicates that tumor suppressor genes are frequently inactivated due to epigenetic changes rather than somatic mutations during cancer development. For instance, Schuebel KE et al has observed a higher frequency of DNA methylation associated gene silencing as compared to those somatic mutation associated gene inactivation in colon cancer (Schuebel et al., 2007). This finding was further supported by integrative genomic analyses in ovarian cancer, which identified the frequency of DNA methylation silenced genes was higher than gene inactivation by somatic mutations (2011). Furthermore, the authors also reported that the high occurrence of DNA methylation was correlated with silencing of various tumor suppressor genes, which led to dysregulation of pathways such as Rb, RAS/PI3K and Notch signaling in ovarian cancer. Dependence of these core oncogenic pathways for cell proliferation and survival allows them to collaborate with genetic mutations in the same pathways, providing “addicted” growth advantages that pomote tumor development. Thus, genome-wide mapping genetic and epigenetic gene silencing might provide new insights into cancer prevention and therapy.

Large-scale initiatives to map somatic mutations and gene expression have revealed a large number of genetic alterations that contribute to inactivation of tumor suppressor in cancer cells (Beroukhim et al., 2010; Ding et al., 2008; Kan et al., 2010; Wood et al., 2007). In addition, epigenetic gene silencing due to DNA

methylation is one of the most common mechanisms for tumor suppressor genes to be inactivated during tumorgenesis (Baylin and Ohm, 2006). Recent efforts in genome-wide studies have also showed that epigenetic regulation of tumor suppressor genes is tightly linked to chromatin modifications, such as histone methylation and deacetylation (Esteller, 2008; Ohm et al., 2007). For example, transcriptionally active tumor suppressor genes are characterized by active chromatin marks, such as trimethylated histone H3 lysine 4 (Mikkelsen et al., 2007; Zhao et al., 2007) . Thus, the restoration of H3K4me3 could be a useful marker for detecting the reactivation of silenced tumor suppressor genes after DNA demethylation treatment. Current technology advancements in high-resolution, genome-wide mapping of DNA methylation and histone modifications including ChIP-PET or ChIP-seq are widely utilized to characterize epigenome modifications during cancer development (Kondo et al., 2008; McGarvey et al., 2008; Meissner et al., 2008; Mikkelsen et al., 2007; Zhao et al., 2007). The advancements in genome-wide technologies provide an excellent platform to carefully characterize and dissect the mechanisms behind epigenome dysregulation in cancer.

In this chapter, a genome-wide approach was adopted to identify genes that are silenced by DNA methylation in colon cancer. Through integrated genomic and epigenomics analysis, A high-throughput strategy was utilized to identify the silenced genes in CRC. The workflow of this study is illustrated in Figure 3.1. Two well-defined isogenic HCT116 and DKO cell lines (HCT116 cells harboring genetic disruptions both DNMT1 and DNMT3B loci (DKO)) were used, Microarray

analysis was performed to compare the differentially regulated genes in HCT116 and DKO or HCT116 treated with DNA demethylation agent, 5-AzaC. Subsequently, hierarchical clustering was performed based on the mRNA expression levels of a panel of colon cancer cell lines along with 24 pairs of colon primary tumors matched with their respective normal colon musco. By overlapping these two sets of genes, a subset of DNA methylation-regulated genes were identified. In addition to above analysis, ChIP-seq analysis was performed to examine the H3K4me3 occupancy in HCT116 and HCT116-DKO to identify the active promoter regions of the tumor suppressor genes. Finally, genes with H3K4me3 marks in DKO cells but not in HCT116 cells were overlapped with the list of cancer related methylated genes to select as a group of colon cancer methylation silencing genes (CMS) for further study. In this study, we have demonstrated that the changes in H3K4me3 signature before and after removal of DNA methylation could lead to the identification of tumor suppressor genes that are epigenetically regulated during cancer progression. Functional analysis of randomly selected CMS genes have demonstrated that the re-expression of these genes could suppress tumorigenicity in colon cancer cells. These observations demonstrate that the integration of genomic and epigenomic studies is an efficient strategy to identify DNA methylated genes that are involoved in cancer development.

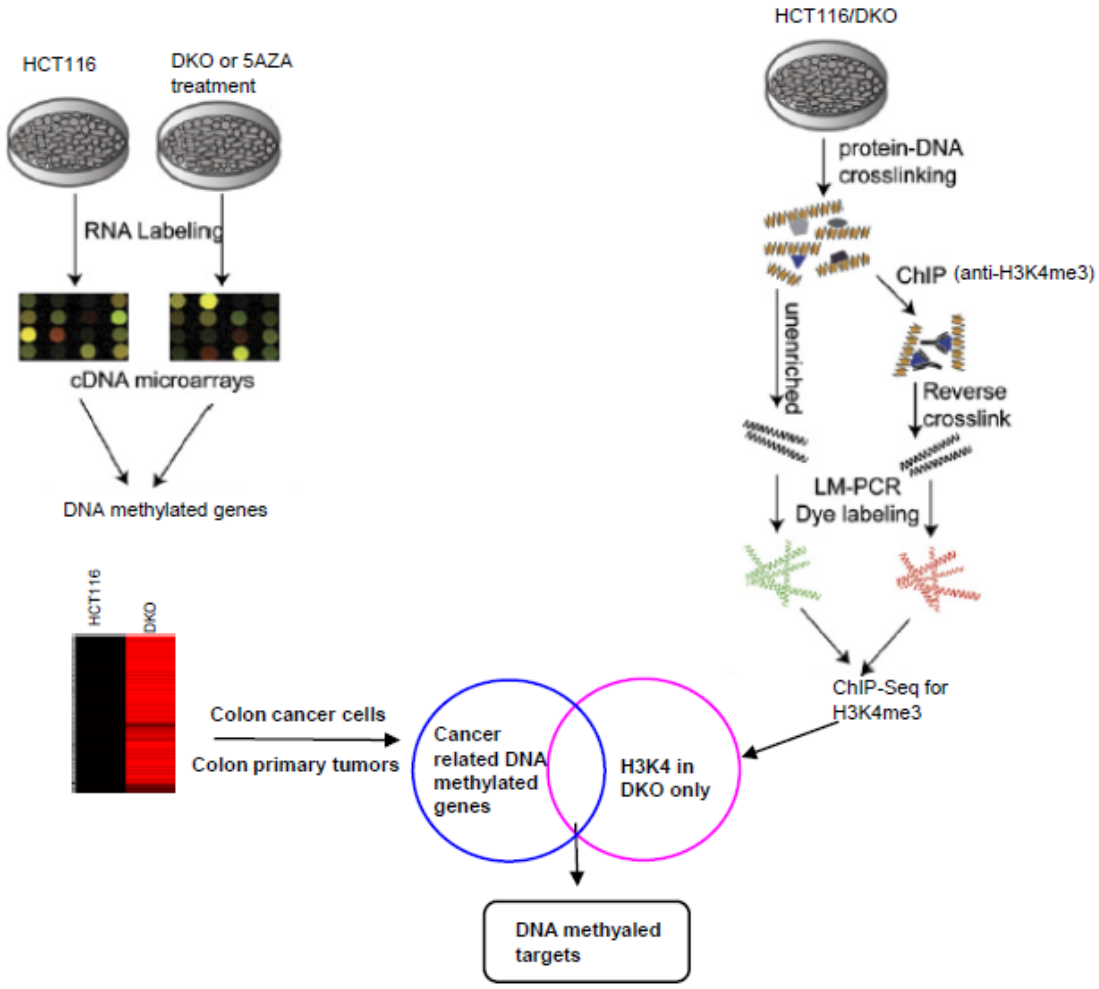


Figure 3.1 Strategy of integrative genomic and epigenomics analysis used to identify DNA methylation targets in cancer.

Transcriptional targets of DNA methylation repression were identified by expression profiling in HCT116 and DKO cells or HCT116 cells treated with DNMT inhibitor 5-AzaC. This DNA methylation repression signature were evaluated for clinical relevance in CRC. In parallel, a cohort of gene promoters occupied by the H3K4 tri-methylation was identified by genome-wide location analysis. The caner related DNA methylation genes that are also physically bound by the H3K4me3 in DKO only were nominated as colon cancer methylation silenced genes (CMS).

3.2 Results

3.2.1 Microarray analysis reveals epigenetically silenced genes by DNA hypermethylation in colon cancer cell lines

The purpose of this study is to characterize silenced genes by DNA methylation. To do this, we first carried out microarray analysis in colorectal cancer cell line HCT116 and its counterpart HCT116-DKO in which both DNMT1 and DNMT3B are genetically disrupted or HCT116 cells treated with DNA methylation inhibitor 5-aza-cytidine (Aza). Changes of mRNA expression in DKO and HCT116 with or without 5-AzaC treatment were analyzed using the Sentrix® HumanRef-8_V2 Expression BeadChip Kit(Illumina, USA). Microarray scanned images were imported to Illumina® GenomeStudio 2010 for data quality control. With GeneSpring GX 11.0.2, the gene expression level data file were transformed to log2 values and quantile normalized. With the normalized gene expression levels, significant genes were selected based on significance analysis of microarrays (SAM) by separately comparing HCT116-DKO or HCT116/5-AzaC with the HCT116 parental control (HCT116). Genes with significant differential expression were selected based on a minimum 3 fold change and FDR (False Discovery Rate) < 0.1%. Using the Illumina BeadChip technology, we identified 1,275 genes with upregulated expression in either HCT116-DKO cells or HCT116 cells treated with 5-aza-dC compared to HCT116 cells (Figure3.1A). Hierarchical clustering analysis revealed 753 genes that was consistently repressed across a panel of colon cancer

cell lines compared to normal colon epithelium (Figure3.1B). These data suggest that DNA methylation at these genes might be used extensively to repress their expression in most colon cancer cell lines.

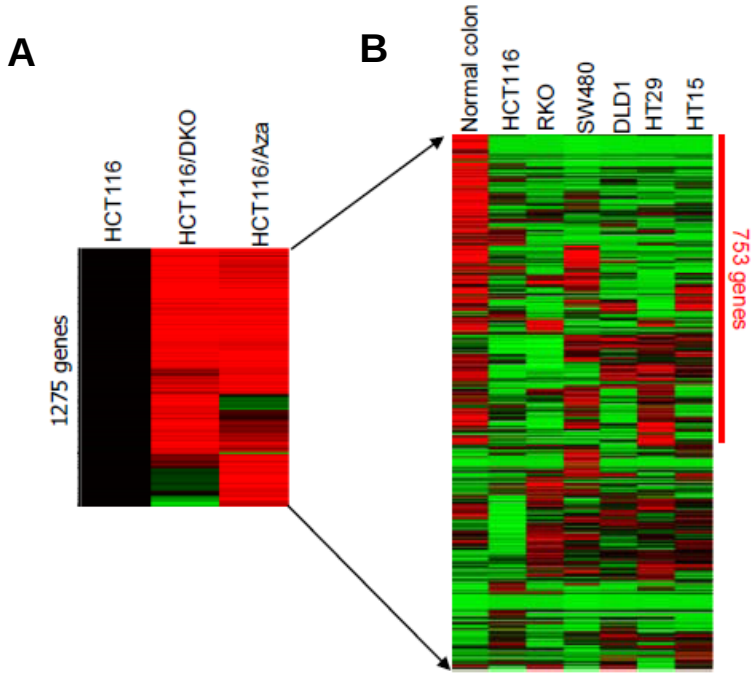


Figure 3.2 Genes silenced by DNA hypermethylation in colon cancer cancer cell lines.

(A) 1275 genes were upregulated with 3 fold or greater in either DKO cells or HCT116 cells treated with 5.0 μM 5-AzaC for 3 days compared to parental HCT116 cells.

(B) Expression profiles of 1275 genes in normal colon tissue and multiple colon cancer cell lines, and 753 genes show consistent downregulation in multiple colon cancer cells compared to normal colon tissue.

3.2.2 Microarray analysis reveals silenced genes in primary colon tumors

As aberrant DNA methylation plays a critical role in human cancer progression, we proceeded to source out for DNA methylation-suppressed genes with clinical relevance. To determine whether the above gene set identified in established cell lines are of clinical relevance, we carried out microarray analysis in colon primary tumor to compare their expression profiles. We subjected the 753 downregulated genes into the microarray database of 24 pairs of clinical colon tumor samples with matched normal colon tissues. The hierarchical clustering analysis revealed that 476 out of 753 genes showed a consistent and marked repression in tumors compared to the matched normal controls, illustrating the clinical relevance of this subset of genes beyond the initial cell lines studied (Figure3.2). Among this 476 gene, some are well-established genes that were previously reported to be silenced by DNA hypermethylation in colon cancer, such as *SFRP1*, *CDH2*, and *UCHL1* (Okochi-Takada et al., 2006; Suzuki et al., 2004).

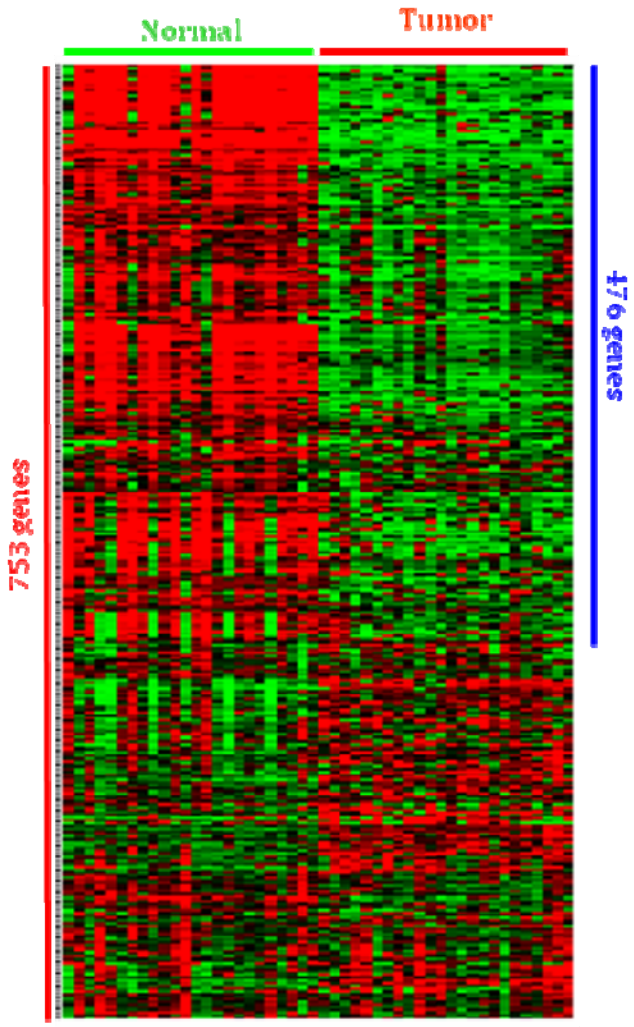


Figure 3.3 476 out of 753 genes show consistent downregulation in human primary colon tumors compared with the normal tissues

3.2.3 Genome-wide mapping H3K4me3 marks in HCT116 and DKO cells

Histone H3 lysine 4 trimethylation (H3K4me3) is considered to be a marker of an active gene promoter (Mikkelsen et al., 2008; Zhao et al., 2007), and the majority of these marks are located within 1.0 kb of transcription start site (TSS). Moreover, H3K4me3 and DNA hypermethylation are mutually exclusive: DNMTs only recognize sites with unmethylated H3K4 (Ooi et al., 2007; Weber et al., 2007) and loss or gain of H3K4 methylation is a strong predictor of promoter DNA methylation status (Meissner et al., 2008). As such, by investigating the H3K4me3 status in our original HCT116 cells and the corresponding DNMTs double knock out line (DKO cells) would provide a measure of promoter activity (and DNA methylation status) that is independent of our gene expression profiling. Using genome-wide microarray analysis, we uncovered 476 genes consistently silenced in both colon cancer cell lines and primary tumors, which were thus associated with clinical relevance. We next examined the H3K4me3 signature of these 476 genes in HCT116 cells and its counterpart HCT116-DKO cells. We performed chromatin immunoprecipitation (ChIP) using antibodies against H3K4me3 in both HCT116 and DKO cells followed by high-throughput sequencing analysis. Thus a genome-wide map of H3K4me3 binding sites in both HCT116 and DKO cells were generated. A total of 11,747 and 13,074 high confidence H3K4me3 sites were identified in HCT116 and DKO cells, respectively, within 1.0 kb of a TSS (using a false discovery rate [FDR] of 0.1%). Overlapping these two data sets revealed 2,507 gene promoters that lacked

H3K4me3 in HCT116 cells but gained this mark in the DKO cells (Figure 3.4).We designated this group of genes as DNA methylation targets, which could be reactivated by DNA demethylation in DKO cells but silenced in HCT116 cells.

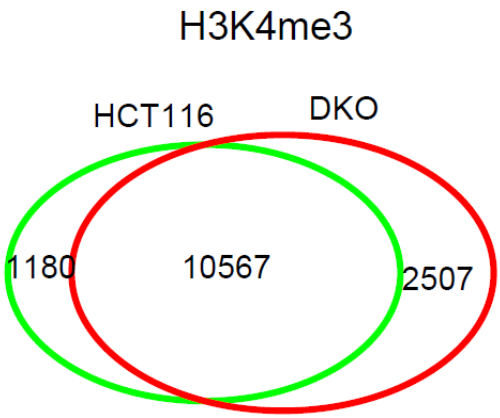


Figure 3.4 Genome-wide analysis of H3K4me3 was done in HCT116 and DKO cells by using ChIP-seq and Solexa Genome Analyzer.

Venn diagram depicts the overlapping of gene promoters marked by H3K4me3 surrounding the transcriptional start site (<1.0 kb) from the two cell lines. 10567 promoters were binded by H3K4me3 as expressed genes in both HCT116 and DKO cells. 1180 promoters were identified as gain of H3K4me3 in HCT116 cells. 2507 promoters were identified to carry high H3K4me3 in DKO cells but not in HCT116 cells.

3.2.4 Identification of cancer methylation silenced genes (CMS)

To distinguish the most robust gene set that appeared to be silenced in colon cancer cells by DNA methylation, we next identified the genes that fell in the union between those genes identified by expression Beadchips and those by H3K4me3 ChIP-seq. By comparing with above-identified 476 DNA methylation silenced genes with 2507 H3K4me3 marks in DKO cells but not in HCT116 cells, we were able to identify a set of 203 genes that appears to be truly silenced by DNA methylation and lack of detectable H3K4me3 in HCT116 cells and they are re-expressed in DKO cells with a gain of H3K4me3 (Figure 3.5). For convenience of the description in the following studies, we define these genes as colon cancer methylation silenced genes (CMS) in the remaining of the chapter.

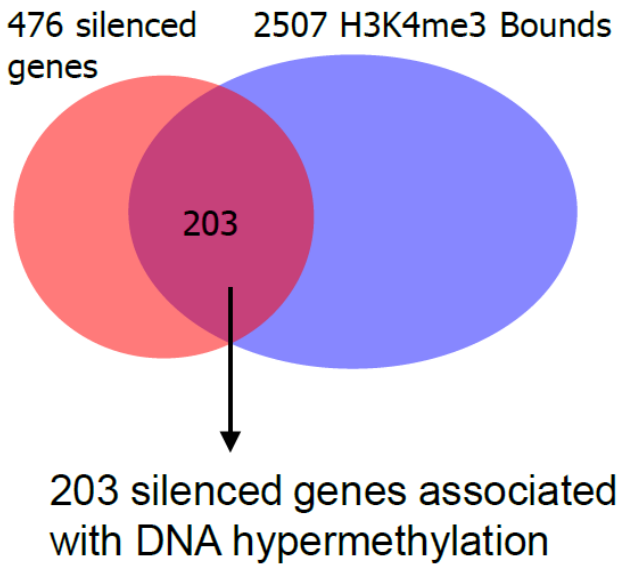


Figure 3.5 203 silenced genes by DNA methylation

Venn diagram depicting an overlap of 203 genes that were repressed by DNA hypermethylation with no detectable H3K4me3 in HCT116 cells, thus defined as genes silenced by DNA methylation.

3.2.5 Validation of cancer methylation silenced genes (CMS)

To examine whether the CMS genes were indeed silenced by DNA methylation in HCT116 cells and reactivated in DKO cells by DNA demethylation and H3K4 tri-methylation. Two well-known genes silenced by DNA methylation were selected for validation. Two CMS genes (*SFRP1* and *CDH2*) illustrated in Figure 3.6 exemplify such changes in DNA methylation and H3K4me3 in HCT116 and DKO cells. RT-PCR results indicated that both *SFRP1* and *CDH2* were reactivated in DKO cell but not in HCT116 cells (Figure 3.6A). Methylation-specific PCR (MSP) was performed to check the DNA methylation status in the promoter. As shown in Figure 3.6B, both *SFRP1* and *CDH2* were hypermethylated in HCT116 cells, which only amplified by methylated-primer (M) but not by unmethylated-primer (U). In contrast, bisulfite DNA was only amplified by unmethylated primer in DKO cells, which indicate DNA promoter was demethylated in DKO cells. At the same time, ChIP-seq data shows that H3K4me3 was only bound on the active promoter of DKO cells but not HCT116 cells (Figure 3.6C). We further examined an additional set of randomly selected CMS genes by RT-PCR and methylation-specific PCR (MSP) (Figure3.7A and 7B). In each case, the gene was verified to be promoter methylated and silenced in HCT116 cells but became demethylated and re-expressed in DKO cells, validating the accuracy of this approach.

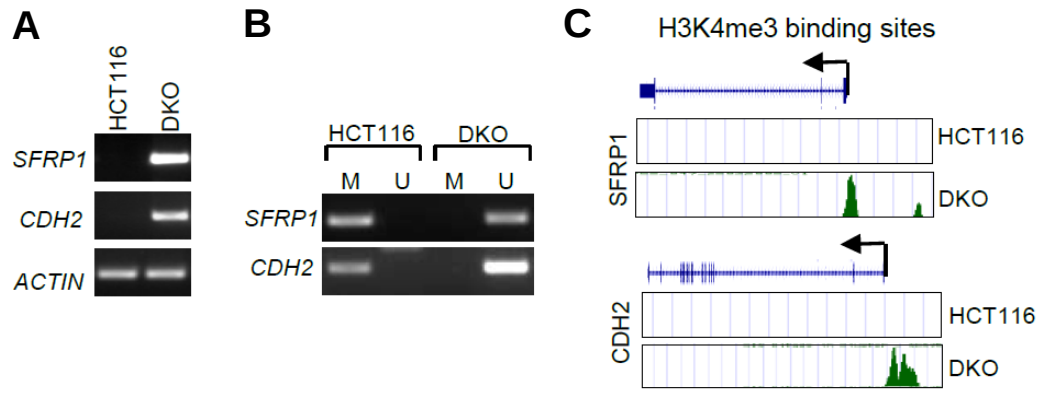


Figure 3.6 Representative genes showing the differential gene expression, methylation status and H3K4me3 in HCT116 and DKO cells.

(A) RT-PCR shows that *SFRP1* and *CDH2* were silenced in HCT116 and re-expressed in DKO cells.

(B) MSP shows that *SFRP1* and *CDH2* promoters were methylated in HCT116 cells but demethylated in DKO cells.

(C) ChIP-seq result shows that H3K4me3 was detected at *SFRP1* and *CDH2* promoters in DKO cells but not in HCT116 cells.

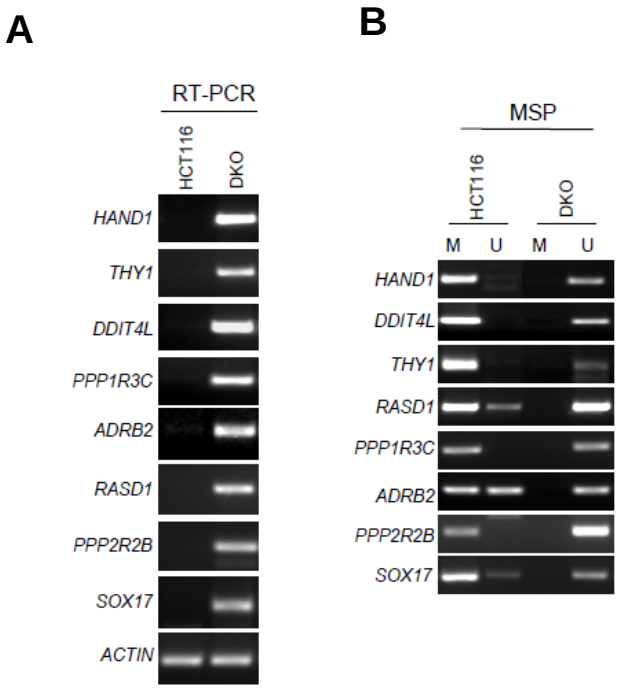


Figure 3.7 Validation of CMS genes in HCT116 and DKO cells.

(A) RT-PCR analysis of random-selected CMS genes in HCT116 and DKO cells.

(B) MSP analysis of the CMS genes as (A) in HCT116 and DKO cells. M, methylated; U, unmethylated.

3.2.6 A global analysis of CMS genes reveals pathways dysregulated in CRC

A pathway analysis revealed that a number of DNA methylation targets were implicated in pathways which have important roles in regulating tumorigenesis (Sjoblom et al., 2006; Wood et al., 2007), and the silencing of these CMS genes would lead to induction of these pathways (Figure3.8). For example, epigenetic repression of extracellular Wnt inhibitors, such as DKK1/3 and SFRP1, has been often reported in human colorectal cancer cells (Baylin and Ohm, 2006), which lead to aberrant Wnt/b-catenin signaling in colorectal cancer cells. To identify novel signaling pathways involved in the loss of CMS genes, we performed an Ingenuity Pathway Analysis using the 203 CMS genes and mapped the pathways onto the heatmap of colon primary tumors. Table 3.1 shows the results of Gene Expression Ontology (GEO) analysis of the top function of CMS genes. These results indicated that many of these CMS genes are involved in cell movement, cell death, and cell proliferation, which were ranked by p-value. Figure 3.8 shows that loss of CMS genes results in activation of known oncogenic pathways in CRC according to the IPA results. The loss of the DNA methylation targets such as *RASD1* and *DDIT4L* leads to the activation of RAS and PI3K/mTOR pathway, respectively. In addition to these WNT inhibitors like *SFRP1* and *DKK1*, *WNT5B* and SRY-box containing gene 17(*SOX17*) were also identified as tumor suppressor genes silenced by DNA methylation, which targets the WNT signaling pathway (Zhang et al., 2008). Among these inhibitors of oncogenic pathways, some are important transcription factors that

were shown to contribute to the regulation of neoplastic process. For example, *HAND1* works as a differentiation transcription factor and functions as a tumor suppressor in melanoma and thoriod carcinomas (Martinez Hoyos et al., 2009; Tellez et al., 2009), which is also silenced by DNA methylation. Taken together, these results suggest that DNA methylation plays as a pivotal mechanism in the loss of CMS genes, and such loss of CMS genes may contribute to activation of oncogenic signaling in colorectal cancer.

.

Table 3.1 The top10 list of GEO results of 203 CMS genes.

	Category	p-value
1	Cellular Movement	3.55E-08-9.56E-03
2	Genetic Disorder	1.12E-07-9.56E-03
3	Cell Death	3.47E-07-9.56E-03
4	Cellular Growth and Proliferation	1.1E-06-8.94E-03
5	Tissue Development	1.67E-06-9.56E-03
6	Cancer	2.46E-06-9.56E-03
7	Reproductive System Disease	2.83E-06-9.56E-03
8	Nutritional Disease	5.23E-06-9.56E-03
9	Cell Signaling	5.93E-06-5.25E-03
10	Nucleic Acid Metabolism	5.93E-06-9.56E-03

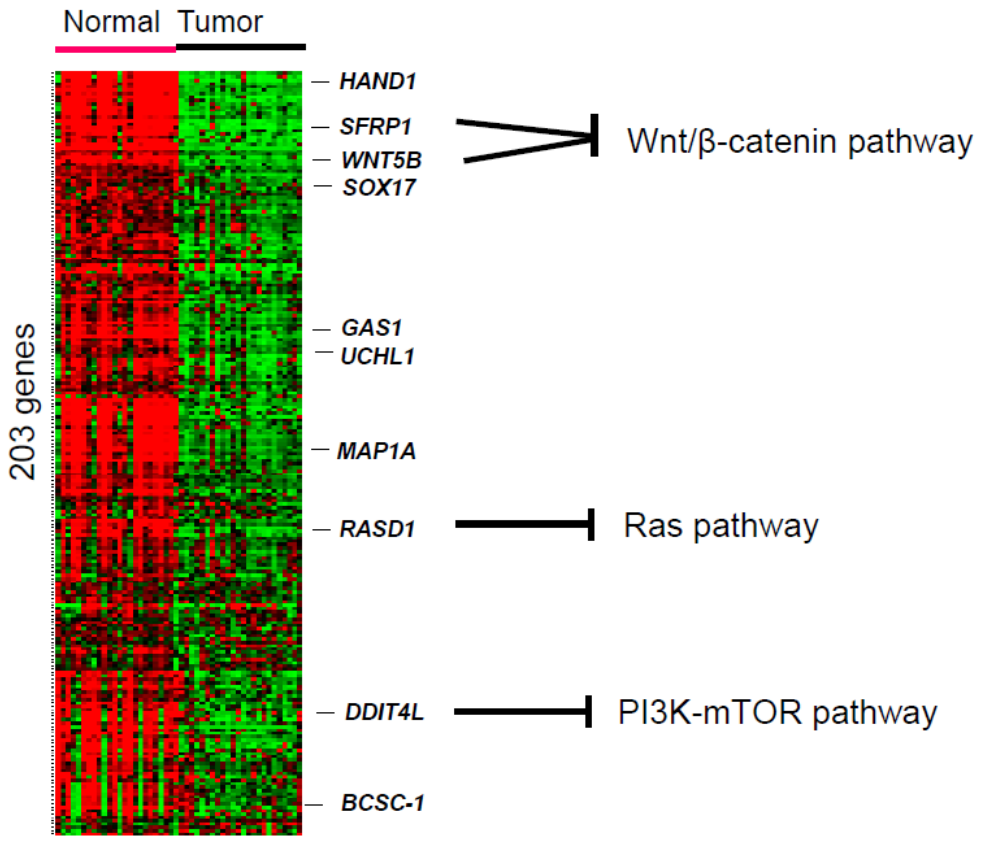


Figure 3.8 Potential oncogenic signaling pathways in colorectal cancer

Potential oncogenic signaling pathways that were involved in the inactivation of tumor suppressor functions of the 203 CMS genes.

3.2.7 Functional validation of CMS genes in colon cancer cells

A subset of CMS genes were previously reported as tumor suppressors in CRC. However, the function of the unknown CMS genes remain elusive. To investigate the tumor suppressive potentials of CMS genes in colon cancer cells, we constructed expression vectors encoding various CMS genes of interest and carried out colony formation assays in cancer cell lines. We focus on three CMS genes (*RASD1*, *HAND1*, and *THY1*) and their ability to function as suppressors of tumor growth in HCT116 and DLD1, in which all three genes were silenced. We tested these three

genes by a colony formation assay in soft agar using zeocin selection after transfection with each gene or control vector (pcDNA4). Figure3.9 showed that the restoration of *HAND1*, *RASD1* and *THY1* genes in both DLD1 and HCT116 cells significantly reduced the colony formation ability in soft-agar assay, which indicated that all three genes have potent tumor-suppressing activity. In generally, these results suggest that re-expression of the CMS genes may suppress the cancer phenotype in colon cancer cells.

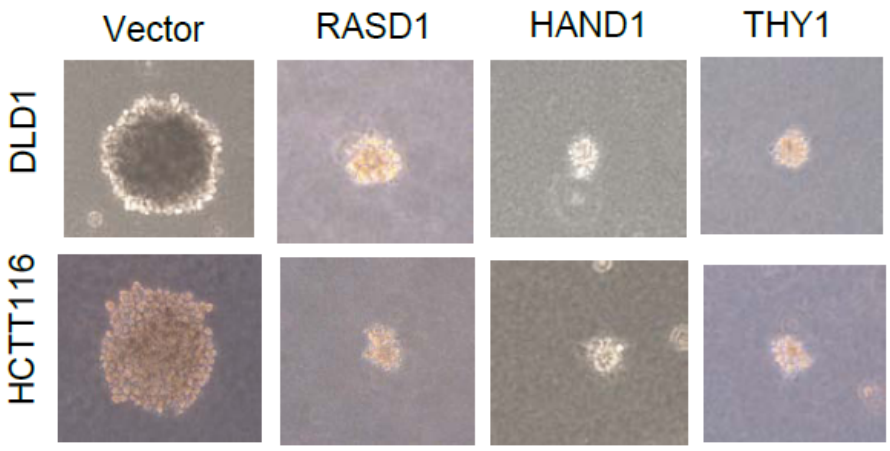


Figure 3.9 Anchorage-independent colony formation assay in soft-agar

Human full-length RASD1, HAND1 and THY1 were cloned in pcDNA4 mammalian expression vector and transfected into both DLD1 and HCT116 cells. transfected cells were seeded for colony formation assay in soft-agar. Representative images were photographed after selection with 100μg/ml zeocin for 14 days.

3.3 Discussion

In this chapter, we used two complementary approaches to harness the power of comprehensive profiling technologies for the identification and validation of silensing tumor suppressor genes in colorectal cancer. Through integrative genomic and epigenomic analysis, we identified 203 novel DNA methylation silencing genes and many of them have been implicated in human malignancies. For example, WNT signaling inhibitors, such as *SFRP1*, *WNT5B*, and *SOX17*, were identified by screening for epigenetically silenced genes in CRC cells, and their methylation was subsequently found in colon primary tumors (Suzuki et al., 2004; Zhang et al., 2008). Epigenetic silencing of these WNT signaling antagonists may collaborate with genetic alterations of β-catenin or APC and contribute to the abnormal activation of WNT signaling pathway in CRC (Baylin and Ohm, 2006). The high frequency of DNA methylation of these tumor suppressor genes in CRC is suggestive of their tumor suppressor function. Functional studies of several CMS genes showed that these CMS genes work as tumor suppressors in colon cancer cells. Moreover, a significant number of CMS genes have also been reported to be silenced by DNA hypermethylation in other cancers, such as *ADRB2*, *THY1* and *RASD1*, which function as tumor suppressors in prostate cancer, nasopharyngeal carcinoma and other cancers (Lung et al., 2005; Yu et al., 2007).

Cancer progression is associated with poor differentiation of cancer cells. A link between DNA methylation event in cancer and differentiation program has not been demonstrated. In this study, we have identified a differentiation transcription

factor *HAND1* that was silenced by DNA methylation in colon cancer and showed that restoration of *HAND1* is able to inhibit colony formation in soft agar. Given that the silencing of *HAND1* occurred in near 90% of colon cancer, we predict that loss of this gene is an important developmental regulator in colon tissue and might play an important role in cancer progression. Moreover, the high frequency of DNA methylation-mediated silencing of *HAND1* indicates that it could be a novel tumor marker for early detection of colorectal cancer. Among the 203 CMS genes, some of them encode protein phosphatases, such as *PPP1R3C*, *PPP1R16B* and PP2A B subunit *PPP2R2B*. Given the importance of protein phosphatases in regulating various kinases in oncogenic pathways, we selected the PP2A B subunits *PPP2R2B* for further functional study which will be discussed in detail in the chapter IV.

In summary, we provide compelling evidence that a large number of DNA methylation genes are extensively silenced by DNA methylation and are strikingly deficient of active histone modification H3K4 tri-methylation in the DNA promoter in CRC. Our data suggest that DNA demethylation can alter the H3K4me3 signature of numerous silenced tumor suppressor genes in cancer, and that re-expression of these genes has important relevance to the effects of epigenetic related cancer therapy.

4 Chapter IV: Functional investigation of *PPP2R2B* as tumor suppressor in CRC

4.1 Introduction

Protein phosphatase 2A (PP2A) belongs to a family of holoenzyme complexes that function in various cellular signaling pathways (Eichhorn et al., 2009; Janssens et al., 2005; Westermarck and Hahn, 2008; Yeh et al., 2004). PP2A dephosphorylates various protein kinases which are involved in multiple oncogenic signaling pathways. Thus, PP2A often functions as a tumor suppressor in human cancer (Mumby, 2007). Mechnistically, PP2A works as a multimeric enzyme that contains the catalytic C subunit, a scaffolding A subunit and one of regulatory B subunits. In mammals, both catalytic C and structural A subnit have α and β isoforms. On the other hand, there are four B subunit families, each with several isoforms or splice variants (Virshup and Shenolikar, 2009). Therefore, variability in the PP2A holoenzyme composition results in the substrate specificity and catalyzes distinct dephosphorylation events imposing different functional outcomes.

Loss-of-function for tumor suppressors leads to cancer due to uncontrolled cell growth and affects the initiation and progression of cancer (Blume-Jensen and Hunter, 2001; Futreal et al., 2004; Stratton et al., 2009). Inactivation of tumor suppressor genes can result from both genetic mechanisms such as mutation and deletion or epigenetic mechanisms such as DNA methylation and histone modification (Baylin and Ohm, 2006; Esteller et al., 2001; Ponder, 2001). Identification of these genes provides insights into the biological processes and pathways underlying tumorigenesis. Although the tumor suppressor role of PP2A has well-established in numerous immortalized human cell lines of different tissue orgins

(Chen et al., 2004; Hahn et al., 1999), the genetic and/or the epigenetic evidence pointing to a PP2A inactivation in human malignancy are largely unexplored. A few studies have shown that somatic mutations in the A subunit of PP2A complex, which led to the loss of B subunit binding, was found to be present in only 15% of lung, breast, and colon cancer (Sablina et al., 2007; Wang et al., 1998), while another study showed that a specific PP2A complex containing B56γ subunit is down-regulated in lung cancer (Chen et al., 2004). Overall, very little is known about how PP2A complexes are altered in human cancer, as well as their effects on cancer signaling pathways and related therapeutic response.

In chapter three, we discovered a set of colon cancer methylation silenced (CMS) genes in human colorectal cancers through integrative genomic and epignomic analysis. Among of these CMS genes, We identified a specific PP2A regulatory B subunit, *PPP2R2B* that was silenced by DNA hypermethylation in human colorectal cancer. In this chapter, we first examined the mRNA expression profiles of all subunits of PP2A in colon cancer cell lines and colon primary tumors to evaluate whether *PPP2R2B* is the only suppressed subunit of PP2A complexes. We hypothesized that *PPP2R2B*-associated PP2A complexes might have a functional role in regulating cancer cell proliferation. Thus, the aim of this project is to identify the underlying mechanism behind the silencing of *PPP2R2B* in colorectal cancer(CRC). Most importantly, we will define the biological functions of this specific *PPP2R2B*-associated PP2A complex and investigate the signaling pathways that are affected by this complex in colon cancer. Furthermore, we hope to identify

the target substreates which could be dephosphorylated upon restoration of PP2A-*PPP2R2B* complex in CRC.

4.2 Results

4.2.1 Loss of *PPP2R2B* expression in colorectal cancer

PPP2R2B is the only PP2A B subunits silenced in CRC lines

Given the low frequency of mutations found in PP2A family members in human malignancy, including colorectal cancer(CRC), we sought to determine whether they are epigenetically inactivated in CRC. In the previous studies, *PPP2R2B* was identified to be epigenetically silenced by DNA hypermethylation (one of 203 CMS genes) in colon cancer cells. To address the role of epigenetic silencing of *PPP2R2B*, the expression profiles of all PP2A subunits was first determined using the Illumina Beadchip gene expression array (Human Ref-8_V2). In a panel of colorectal cancer cell lines and one normal colon mucosa examined, *PPP2R2B* gene that encodes for B55β is the only subunit that is consistently downregulated or silenced in all examined CRC cell lines, but not in the normal colon mucosa samples (Fig4.1A and Table4.1). This finding was subsequently validated by semiquantitative RT-PCR using primers specifically targeting *PPP2R2B* in a panel of CRC cell lines and normal colon tissues. The RT-PCR results showed that *PPP2R2B* was expressed in the normal colon tissues but silenced in all the CRC cell lines (Fig4.1B). In contrast, *PPP2R2A* (B55α) and *PPP2R5C* (B56γ), which has been previously reported to be downregulated in lung cancer (Chen et al., 2004), were not downregulated in CRC.

Taken together, these results demonstrated that the loss of *PPP2R2B* was observed across all CRC cell lines as compared to normal colon tissue.

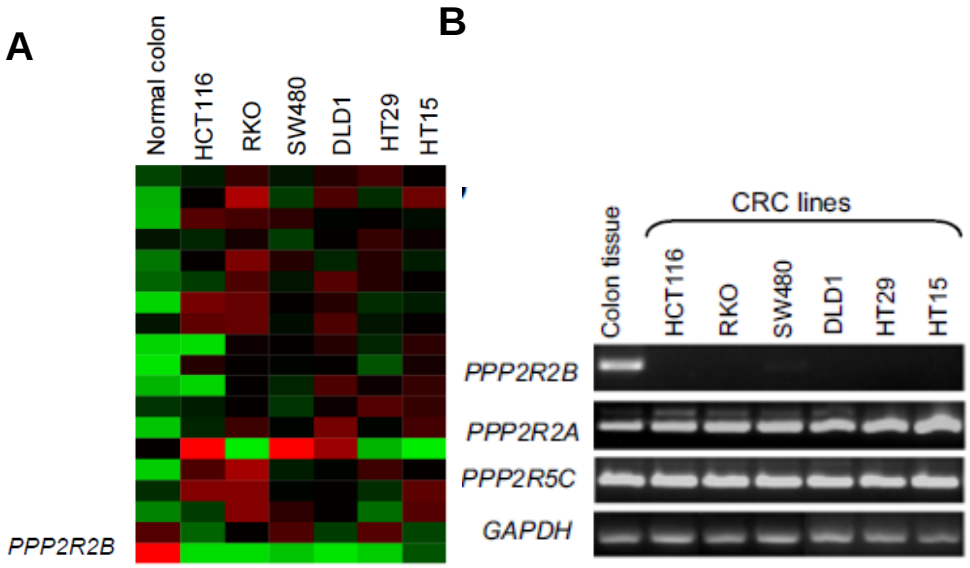


Figure 4.1 PPP2R2B gene is suppressed in CRC cell lines but not in normal colon tissue

(A) Hierarchical clustering of PP2A subunits in human colorectal cancer cell lines. (B) RT-PCR analysis of *PPP2R2B* expression in colon cancer cell lines and normal colon tissues.

Table 4.1 Expression profiles of PP2A subunits in CRC lines and normal colon tissue

Gene name	Accession number	normal colon	HCT116	RKO	SW480	DLD1	HT29	HT15
PPP2R1A	NM_014225	6098	3864.3	6664	7490.5	6629.8	8555.6	6807.8
PPP2R5B	NM_006244	23.7	21.9	30.7	23.3	26.9	17.2	20.8
PPP2R3B	NM_013239	59.3	25.9	241	352.8	292.1	232.9	340.2
PPP2R4	NM_021131	1976	1964.6	5400.6	4031.7	2323.4	2982.3	2649.3
PPP2R2B	NM_004576	436.7	-0.7	0.7	3.1	-7.5	1.8	7.4
PPP2R1B	NM_002716	60.8	129	235.7	273.3	420.5	205.7	311.5
PPP2R2A	NM_002717	802.5	1296.8	5271.6	1778.7	2696.8	1514.6	3800.7
PPP2CA	NM_002715	3782	10967.4	11699.9	14805.8	9831	9957.5	10316
PPP2R5D	NM_006245	749.3	449.7	626.9	724.8	708.6	1092.6	944.4
PPP2CB	NM_001009552	64.8	55.6	210.5	150.2	99.3	56.2	170
PPP2R2D	NM_001003656	367.6	242.1	553.6	528.2	663.9	491.3	489
PPP2CB	NM_001009552	1478	2330.6	2933.3	1769.4	1338.5	1106.8	2522.3
PPP2R3A	NM_002718	401.3	85.7	171.8	361.7	147.4	294.1	156.5
PPP2R2C	NM_020416	22	334.6	-15.7	597	48.2	4.9	-15.4
PPP2R5C	NM_002719	259	1522.5	1693.9	2450.9	1900.6	1303.6	2202.6
PPP2R3B	NM_013239	60.3	16.9	141.6	178.9	227.3	162.7	209.6
PPP2R5E	NM_006246	386.9	2439.2	2671.1	2237.4	1826.7	1392.6	1644.9
PPP2R5A	NM_006243	870.1	423.2	645	685.9	692	848.2	783.9

PPP2R2B is frequently silenced in colorectal primary tumors and other human malignancies

To identify the clinical evidence linking *PPP2R2B* expression in colon cancer, the *PPP2R2B* expression was examined in 24 pairs of colon primary tumors and matched normal controls using box-plot analysis. The data showed that *PPP2R2B* was significantly downregulated in patient-derived CRC tumors compared to matched normal mucosa controls ($p < 0.001$, Figure 4.2A). Moreover, the loss of *PPP2R2B* appeared to occur in more than 90% of CRC samples when we clustered all the PP2A subunits in the 24 pairs of CRC tumors and matched normal samples (Figure 4.2B). The downregulation of *PPP2R2B* in colon primary tumors was further verified by perfoming RT-PCR on randomly-selected colon tumor samples and their matched normal adjacent tissues (Figure4.2C). In contrast, PPP2R2A and PPP2R5C were not downregulated in CRC. These data demonstrated that *PPP2R2B* is the only silenced subunit of PP2A complex in colon cancer and the downregulation of *PPP2R2B* expression has highlighted a crucial role of *PPP2R2B* in colon cancer development. In addition, the data mining in the oncomine database revealed that *PPP2R2B* is downregulated in a variety of other human tumors including bladder, brain and esophagus cancer, indicating its frequent inactivation in human malignancy (Figure 4.3). Thus, these results from cancer cell lines and clinical data suggested a potential tumor suppressor role of *PPP2R2B* in tumorigenesis.

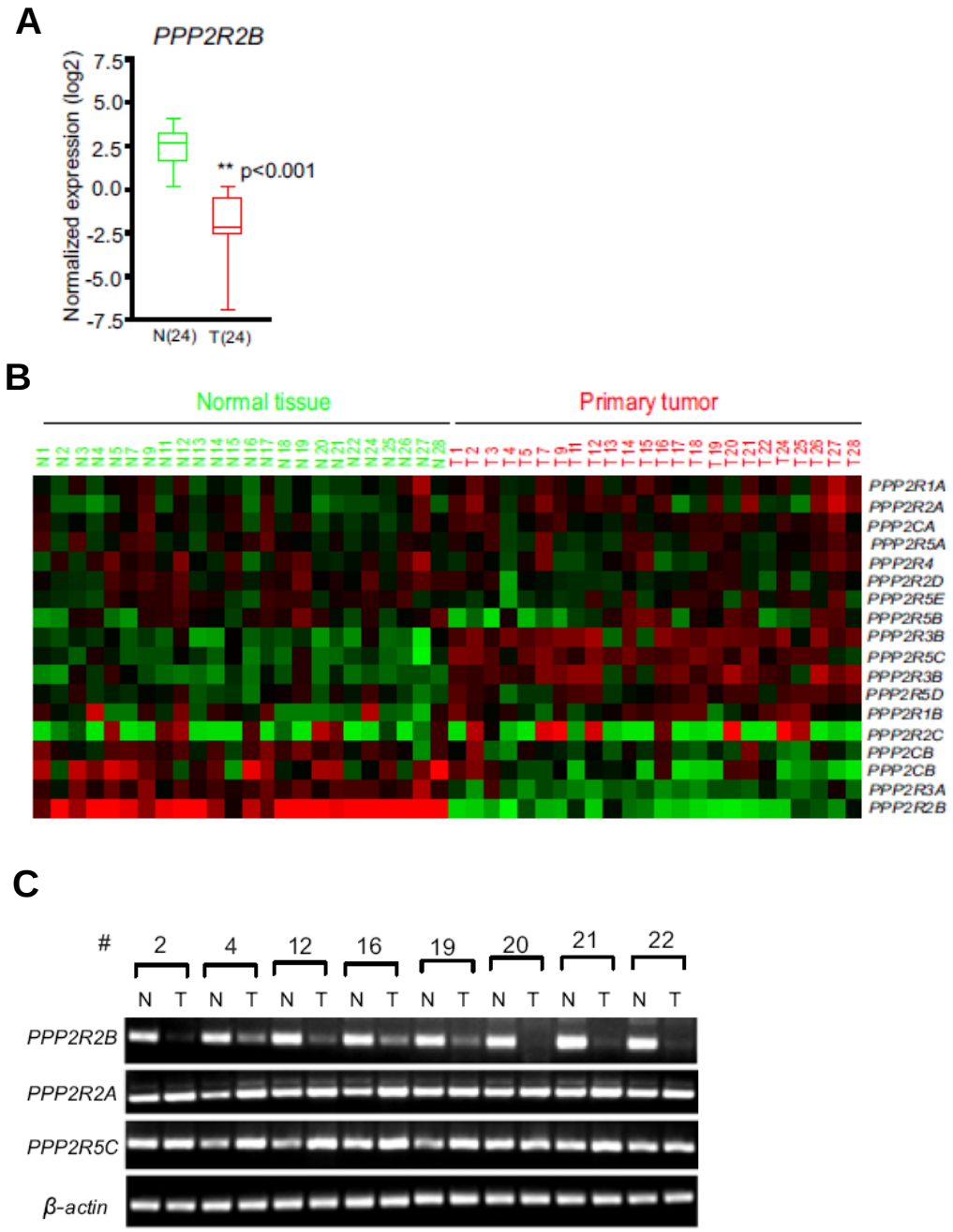
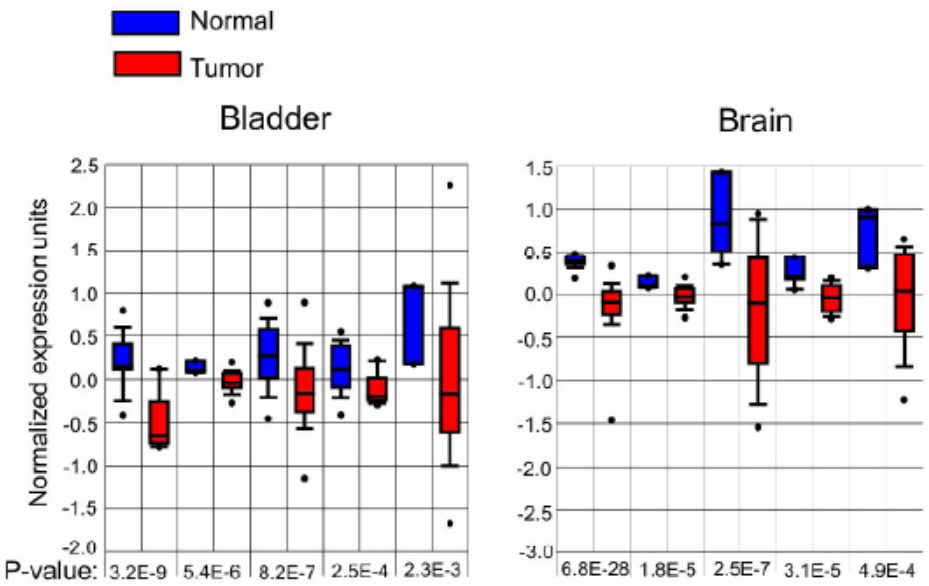


Figure 4.2 PPP2R2B gene is suppressed in colon tumor.

(A) Box-plot showing the differential expression of *PPP2R2B* mRNA levels in 24 pairs of patient-derived CRC and matched normal colon mucosa as determined by Illumina array analysis. P value for difference between tumor and normal is indicated.

(B) Hierarchical clustering of all PP2A subunits in human colorectal tumors (T) and matched normal mucosa (N).

(C) RT-PCR analysis of *PPP2R2B* expression from eight randomly selected pairs of human colorectal tumor (T) and matched mucosa (N). β-actin was used as internal control.



C

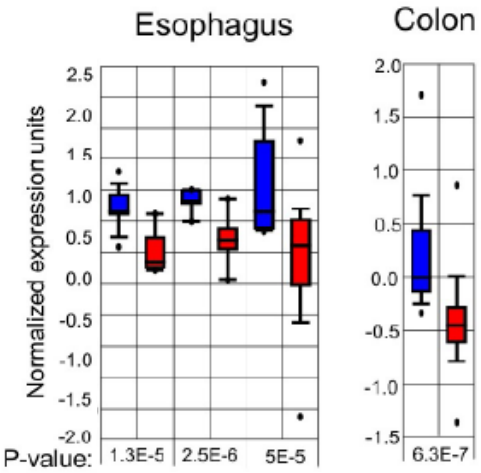


Figure 4.3 PPP2R2B is downregulated in human cancers

Box-plots showing the differential expression of *PPP2R2B* mRNA in tumor and corresponding normal tissues of bladder (Dyrskjot et al., 2004; Sanchez-Carbayo et al., 2006), brain (Rickman et al., 2001; Shai et al., 2003; Sun et al., 2006), esophagus (Wang et al., 2006) and colon (Ki et al., 2007). Representative data were shown across several independent published microarray studies from the ONCOMINE website. The y-axis represents normalized expression and the p-values for correlation are shown below.

4.2.2 *PPP2R2B* is silenced by DNA hypermethylation

PPP2R2B is silenced by DNA methylation in CRC

It is well established that aberrant CpG methylation in the DNA promoter is related to gene silencing in cancer development. We next determined whether the loss of *PPP2R2B* expression in CRC is associated with promoter DNA hypermethylation. In order to identify the silencing mechanism of *PPP2R2B*, methylation-specific PCR (MSP) was used to survey the methylation status of *PPP2R2B* promoter in primary tumor samples. A predicted CpG island was identified in the immediate upstream of the genomic region of *PPP2R2B* gene by using bioinformatics analysis (Figure 4.5C). MSP primers were designed in the CpG island of promoter region according to the bisulfite DNA sequence, which designated with M (Methylated primer) and U (Unmethylated primer). MSP was performed by using methylated primers or unmethylated primers, respectively. MSP results showed that *PPP2R2B* was found methylated in all 8 primary tumors but unmethylated in matched control normal colon tissue (Figure 4.4A). Similar with the primary tumor samples, we also analyzed the the methylation status of *PPP2R2B* in a panel of colon cancer cell lines and non-transformed epithelial cells. MSP results showed that cell lines with silenced expression had methylated promoters compared to normal colon tissue (Figure 4.4B). No methylation was detected in non-transformed epithelial cells (Figure 4.4C). These data support the notion that *PPP2R2B* methylation was correlated with its transcriptional silencing in colon cancer.

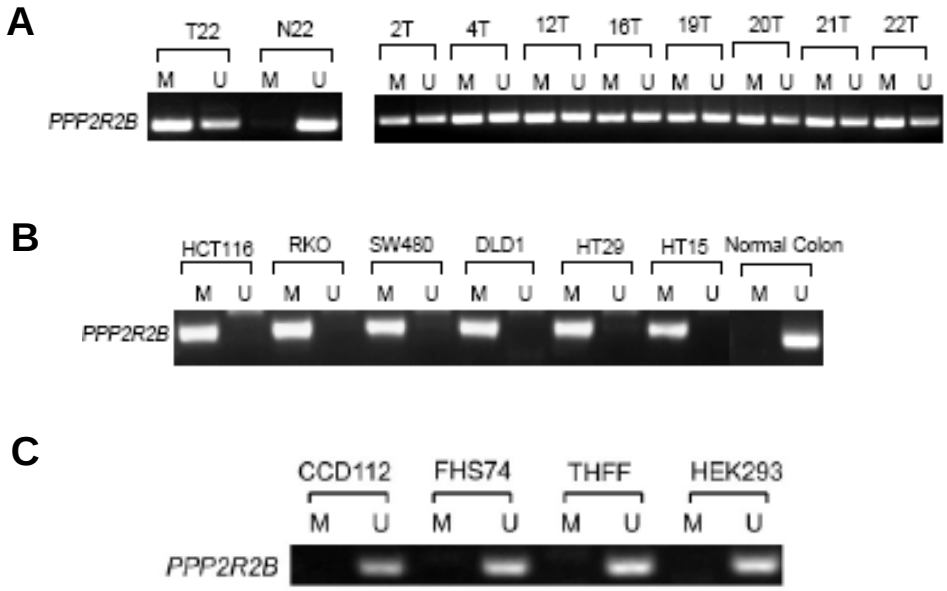


Figure 4.4 PPP2R2B was silenced by DNA promoter hypermethylation in colon cancer.

(A) MSP analysis of *PPP2R2B* promoter in eight selected tumors and matched normal mucosa.
(B) Methylation status of *PPP2R2B* in colorectal cancer cell lines and normal colon tissue.
(C) MSP analysis of *PPP2R2B* promoter in 4 non-transformed cell lines.

PPP2R2B is reactivated by demethylation in the promoter

DNA promoter methylation is mediatated by DNA methyltransferases (DMNTs), such as DNMT1 and DNMT3B (Okano et al., 1999). In recent years, many reports have shown that pharmacologic or genetic disruption of DNA methylation in cancer cell induces upregulation of a substantial number of tumor suppressor genes (Herman and Baylin, 2003; Jones and Baylin, 2007). Indeed, colon cancer cell line HCT116 with the DNA methyltranferase genes DNMT1 and DNMT3B depletion (DKO cells) showed a re-expression of *PPP2R2B* (Figure 4.5A left). Similarly, DLD1 cells treated with DNMT inhibitor 5-aza-dC resulted in an increase in *PPP2R2B* expression (Figure 4.5A right). In agreement with the restoration of *PPP2R2B* expression in DKO cells, MSP results indicated the demethylation of the promoter in DKO cells compared to the hypermethylated promoter in HCT116 cells (Figure4.5B). The detailed methylation status of individual CpG sites in the promoter was further examined by bisulfite genomic sequencing (BGS) analysis in normal colon tissue and HCT116 or DKO cells. The results were consistent with those of MSP (Figure4.5C) indicating that an epigenetic-mediated silencing of *PPP2R2B* in cancer cells may be implicated in tumorigenesis as a potential tumor suppressor role.

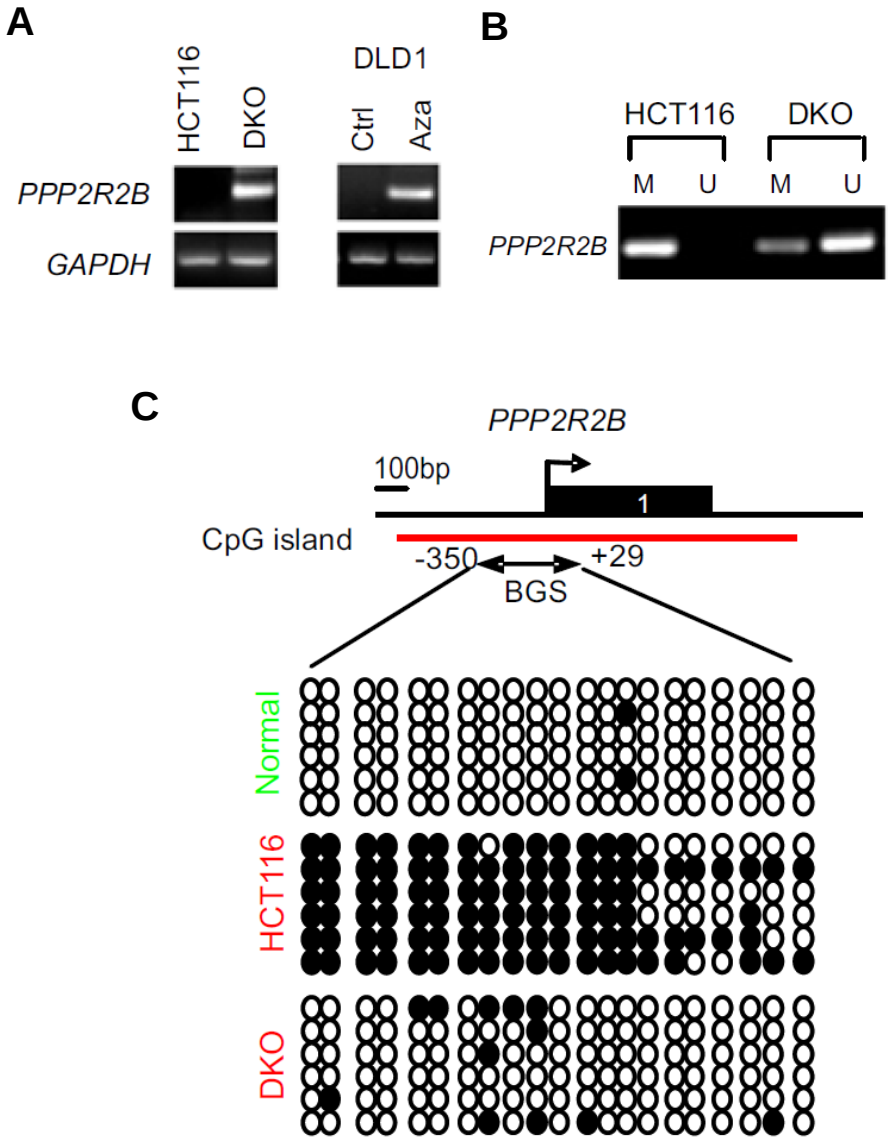


Figure 4.5 PPP2R2B is reactivated by demethylation in DNA promoter

(A) RT-PCR analysis of *PPP2R2B* in HCT116 and DKO or DLD1 cells treated with or without 5-AzaC (5 μM) for 3 days.
(B) MSP analysis of *PPP2R2B* promoter in HCT116 and DKO cells. M: methylated; U: unmethylated.
(C) Methylation analysis of *PPP2R2B* promoter by bisulfite-sequencing analysis (BGS). The region analyzed is indicated. The arrow indicates the transcriptional start site. Open circles represent unmethylated CpGs; closed circles denote methylated CpGs.

4.2.3 *PPP2R2B* functions as a tumor suppressor in CRC

PPP2R2B restoration in colon cancer cells inhibits colony formation

Loss of *PPP2R2B* expression may impair the tumor suppressor activity of PP2A in colon cancers. This hypothesis predicts that cells with defective *PPP2R2B* function may be sensitive to the reconstitution of PP2A complex by exogenous re-expression of *PPP2R2B*. To investigate the tumor suppressor properties of *PPP2R2B*, we examined this possibility by ectopically expressing *PPP2R2B* with a mammalian expression vector in two colon cancer cell lines that do not express *PPP2R2B* (DLD1 and HCT116). Subsequently, functional assays was conducted to detect phenotypic changes in cell viability and anchorage-independent growth. Ectopic expression of *PPP2R2B* inhibits colony formation in both HCT116 and DLD1 cells (Figure4.6A). The strong growth inhibitory effect of *PPP2R2B* was further substantiated in two additional CRC cell lines by using retroviral transduction of both GFP and *PPP2R2B*s, which led to an inhibition of growth in soft agar. As shown in Figure 4.6B, restoration of PP2A-*PPP2R2B* complex significantly reduced anchorage-independent growth rate in both SW480 and HT29. These results indicated that ectopic expression of *PPP2R2B* inhibits colony formation in CRC cells.

Establishment of Tet-on inducible expression cell system

To further elucidate the function of *PPP2R2B* as a potential tumor suppressor, we generated a Dox-inducible expression system in both DLD1 and HCT116 cells expressing either the wild-type *PPP2R2B* or empty vector as negative control. A

brief workflow of Tet-on inducible expression system was shown in Figure 4.7A. After selection by using blasticidin and zeocin antibiotics, single clones were picked up and amplified for screening by immunoblotting analysis of *PPP2R2B* with Myc tagged anti-Myc antibody (9E10). As shown in Figure 4.7B, two clones with lowest background and strongest induction by doxycycline were seleted for functional analysis (clone #6 and #11).

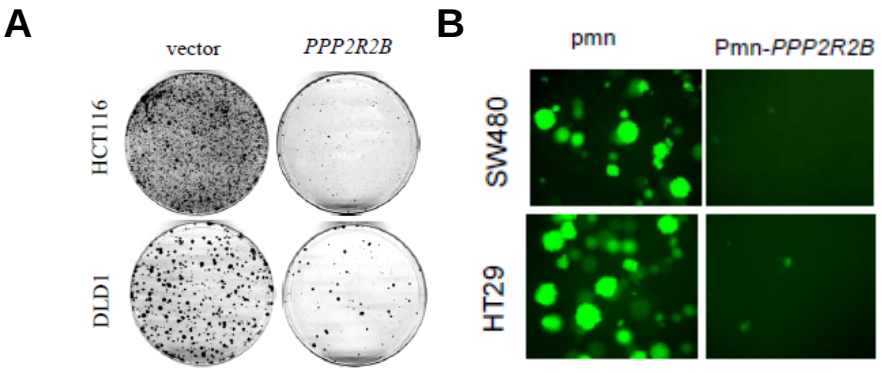


Figure 4.6 Restoration of PPP2R2B in colon cancer cells inhibits cell proliferation and anchorage-independent growth.

(A) Foci formation assay in DLD1 and HCT116 cells with transient expression of *PPP2R2B* and empty vector. $1x10^4$ cells were seeded in 6 well plate and selected with 100µg/ml Zeocin for 14 days.
(B) Anchorage-independent growth assessed by soft agar assay in SW480 and HT29 cells infected with retroviral *PPP2R2B* expression vector. GFP positive cells were seeded in soft-agar and photograph after 14 days.

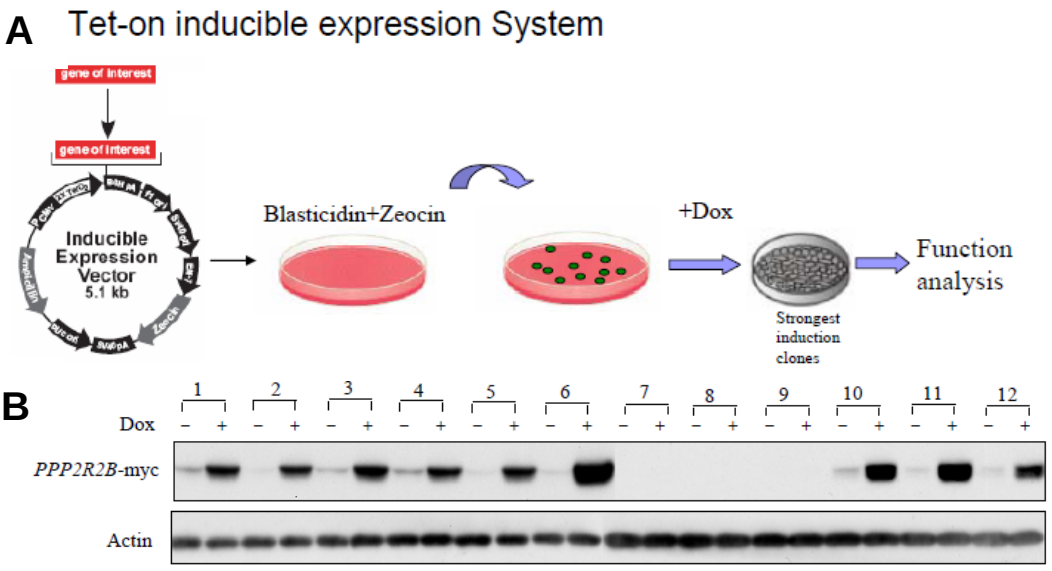


Figure 4.7 Generation of Tet-on inducible expression cell system

Figure 4.7 Generation of Tet-on inducible expression cell system
(A) Workflow of construction of Tet-on inducible expression cell system.
(B) Representative immunoblotting analysis of screening for stable clones with *PPP2R2B* in DLD1 cells.

Gain-of-function analysis of *PPP2R2B* in inducible cell system *in vitro*

To further evaluate the tumor suppressor effects of *PPP2R2B*, we established stable DLD1 and HCT116 cell lines with doxycycline inducible expression of a Myc-tagged *PPP2R2B*, designated as DLD1-*PPP2R2B* or HCT116-*PPP2R2B*. As shown in DLD1-*PPP2R2B* cells, addition of Dox resulted in *PPP2R2B*-Myc (B55β-Myc) expression at levels comparable to the endogenous expression of other three B55 subunits that were not silenced in DLD1 cells, using an antibody that recognizes a total four B55 subunits (α, β, γ, and δ)(Figure 4.8A). The results show that B55β-Myc is induced in CRC cells at a physiological level. Under this condition, ectopic expression of *PPP2R2B* resulted in a typical senescence phenotype, including enlarged and flattened cell morphology, as well as increased senescence-associated β-galactosidase (SA-β-Gal) (Figure 4.8B). Accordingly, DNA synthesis as measured by BrdU incorporation decreased markedly after *PPP2R2B* re-expression by doxycycline treatment(Figure 4.8C), indicative of inhibition of cell proliferation. Furthermore, colony formation assay in soft-agar was performed in both HCT116 and DLD1 inducible cell system in the presence or absence of doxycycline. The results showed that *PPP2R2B* restoration caused a strong inhibition of anchorage-independent growth in both HCT116- and DLD1-*PPP2R2B* cells compared to the control vector cells(Figure 4.8D). Taken together, these results suggest that restoration of *PPP2R2B* inhibits the cancer properties in CRC.

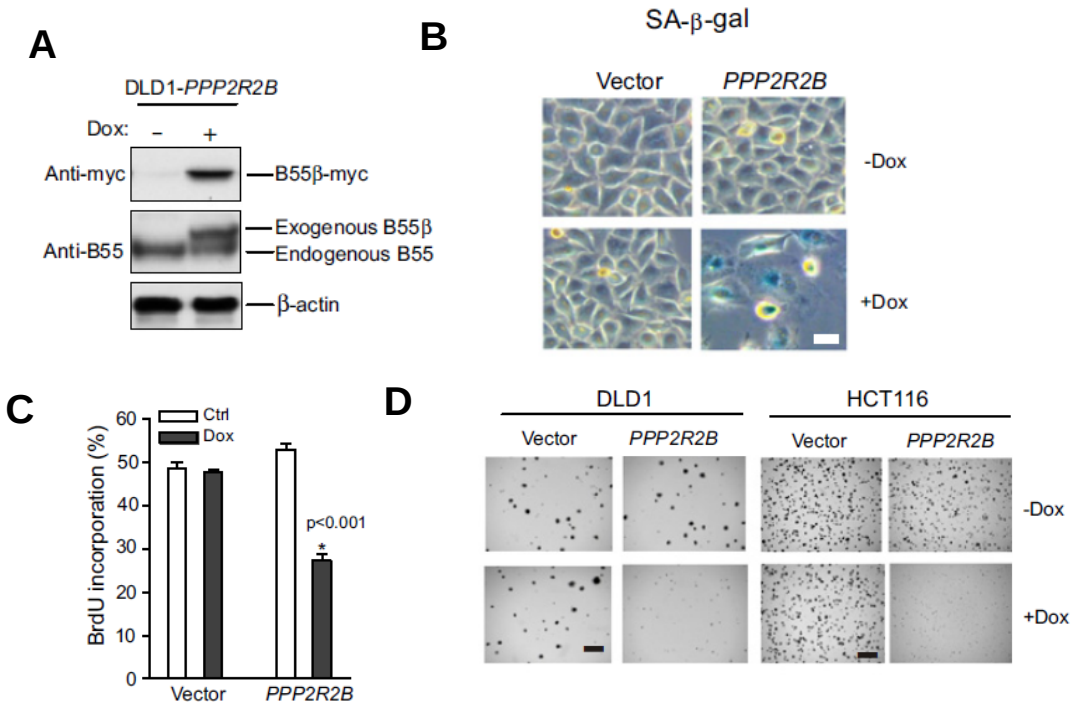


Figure 4.8 Restoration of PPP2R2B results in senescence, decreased cell proliferation and strong inhibition of anchorage-independent growth

(A) Immunoblot analysis of DLD1-*PPP2R2B* cells on exogenous B55β and endogenous B55 using anti-Myc tag or anti-B55 subunit antibody. Cells were treated with or without 10 ng /ml doxycycline for 3 days.
(B) SA-β-gal assay of DLD1-*PPP2R2B* or vector control cells treated with or without Dox for 96 hr. Scale bars, 100 μm.
(C) BrdU incorporation of DLD1-*PPP2R2B* or vector control cells treated with or without Dox for 96 hr. Shown is mean ± SD of three independent experiments.
**$p < 0.001$.
(D) Anchorage-independent growth assessed by soft agar assay in DLD1-*PPP2R2B* or HCT116-*PPP2R2B* cells, treated with or without Dox for 12 days. Scale bar, 10 μm.

PPP2R2B re-expression blocks tumorigenicity *in vivo*

After demonstrating that *PPP2R2B* could function as a tumor supressor of colon cancer cell lines in vitro, the capacity of *PPP2R2B* in tumor growth inhibition *in vivo* was subsequently assessed. Dox-inducible DLD1 cells expressing *PPP2R2B* or the empty vector were subcutaneously injected into nude mice and tumor growth was monitored for 3 weeks with or without daily Dox administration. The data showed that the tumor growth derived from DLD1-*PPP2R2B* cells was markedly inhibited in mice treated with Dox, whereas the tumor growth from the DLD1 cells expressing the control vector was slightly unaffected by Dox treatment (Figure 4.9). The result confirmed the growth inhibitory activity of *PPP2R2B* in the xenograft mouse model. Taken together, our *in vitro* and *in vivo* assays indicate that *PPP2R2B* works as a tumor suppressor in CRC.

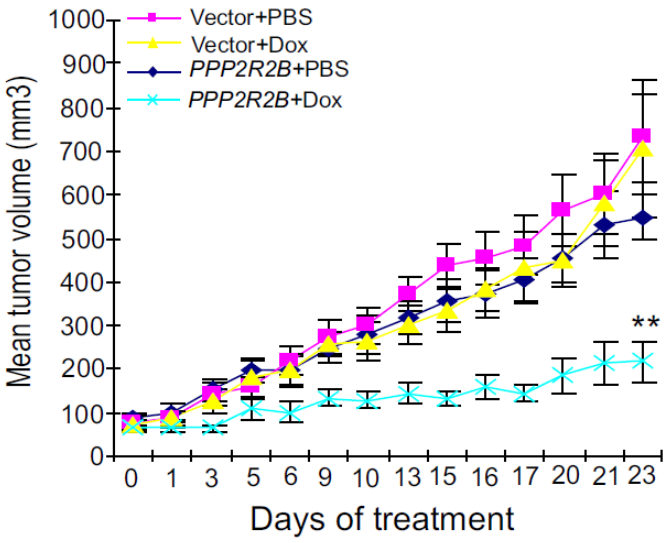


Figure 4.9 Restroration of PPP2R2B in DLD1 cells inhibits tumorigencity in xenograft mouse model

Xenograft tumor growth of DLD1-*PPP2R2B* or control cells in nude mice treated with Dox at 100 mg/kg daily. Error bars represent mean ± SD (n = 8 per group). **p < 0.01 (independent t test).

4.2.4 *PPP2R2B* knockdown promotes cell transformation

We next addressed whether ablation of *PPP2R2B* is sufficient to promote cell transformation in human embryonic kidney fibroblasts, in which SV40 large antigen (LT), hTERT and oncogenic H-RAS (HEK-TERV) are expressed to immortalize but not sufficient to induce tumorigenicity (Chen et al., 2004). In this previously well-characterized transformation model, inhibition of PP2A by overexpression of small T antigen (ST) results in efficient transformation (Chen et al., 2004; Hahn et al., 2002; Sontag et al., 1993). Small interfering RNAs (siRNA) targeting different regions of *PPP2R2B* were transfected into HEK-TERV cells and harvested for qPCR to evaluate the efficiency of siRNA-mediated gene silencing (Figure 4.10A). As shown in Figure 4.10B, siRNA-directed knockdown resulted in an enormous increase in anchorage-independent growth of HEK-TERV cells compared to the control cells transfected with non-targeting siRNA(NC). ST was introduced into HEK-TERV cells as positive control for cellular transformation (Figure 4.10B). To further confirm the results of *PPP2R2B* knockdown, a retroviral shRNA vector pSIREN-zGreen targeting *PPP2R2B* was stably expressed in HEKT-TERV cells and a similar result was also obtained in HEK-TERV cells expressing a stable shRNA targeting *PPP2R2B* compared to non-targeting shNC(Figure 4.10C). On the basis of both the gain- and loss-of-function data, we propose that loss of *PPP2R2B* facilitates oncogenic transformation.

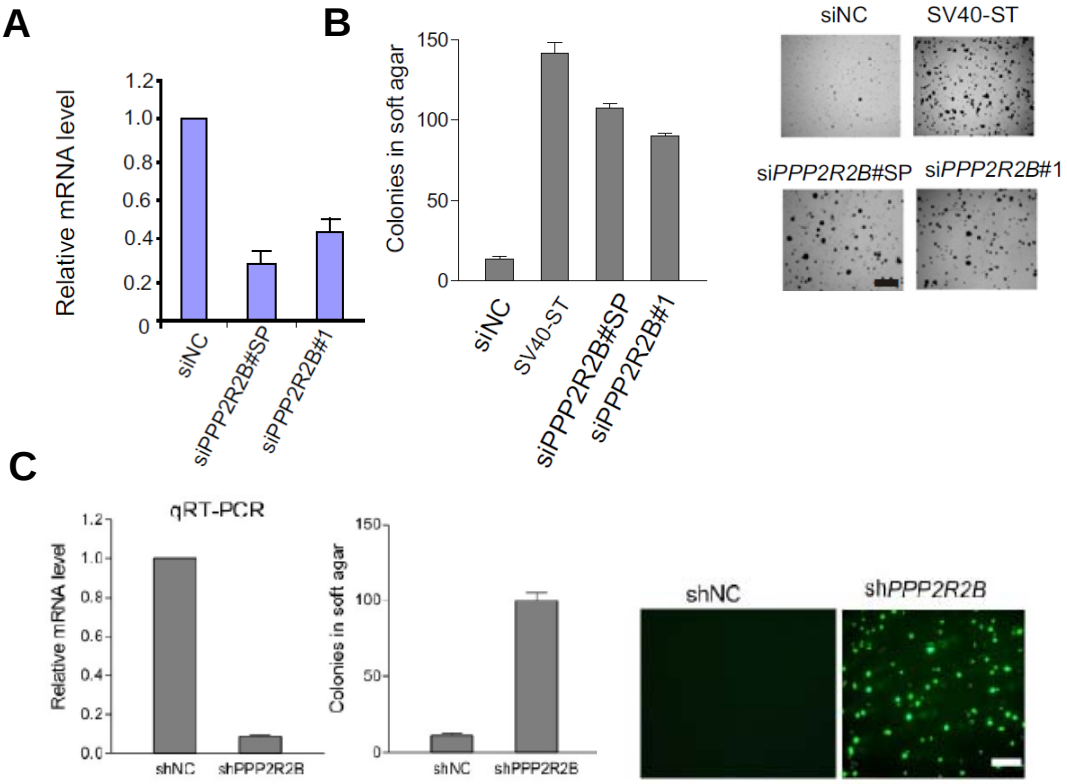


Figure 4.10 PPP2R2B knockdown in epithelial cells promotes cellular transformation

(A) HEK-TERV cells were transfected with a *PPP2R2B* smart pool siRNA or an independent *PPP2R2B* siRNA and the *PPP2R2B* mRNA was assessed by TaqMan assay

(B) Colony formation capacity was assessed by soft-agar assay. Shown on the left is quantification of number of colonies after 14 days from three independent experiments. The representative images of three independent experiments are shown on the right. SV40 small antigen (ST) expressing HEK-TERV cells were used as positive control. Scale bar, 10 µm. Error bar shows mean ± SD of triplicates.

(C) HEK-TERV cells were infected with a *PPP2R2B* shRNA and negative control shRNA and the *PPP2R2B* mRNA was assessed by qRT-PCR (left panel). The error bar shows the mean ± SD of triplicates. Numbers of colonies are presented as the mean ± S.D. of three independent experiments (middle). Representative microscopy images were shown in the right panel. Scale bars =10µm.

4.2.5 *PPP2R2B*-associated PP2A complex modulates phosphorylation of c-Myc and p70S6K in colon cancer cells.

Oncogenic signaling pathways affected by *PPP2R2B* re-expression in CRC

The PP2A B regulatory subunits determine the substrate specificity towards the dephosphorylation events in a cell and context-dependent manner (Virshup and Shenolikar, 2009). Several oncogenic proteins, including AKT, S6K and Myc, have been identified as the substrates of PP2A in various cell systems (Arnold and Sears, 2008; Yeh et al., 2004). To identify the potential oncogenic events in colon cancer cells affected by *PPP2R2B* re-expression which leads to the growth inhibition, we probed the molecular changes in several known oncogenic pathways in HCT116-*PPP2R2B* cells treated with or without Dox for 48 hrs (Figure 4.11). Two phosphorylation events were found to be inhibited following *PPP2R2B* re-expression by Dox treatment, which were p70S6K T421/S424 and Myc. P70S6K T421/S424 was dephosphorylated while mTORC1-induced S6K T389 phosphorylation was not affected upon *PPP2R2B* re-expression, suggesting a site-specific modulation of p70S6K by *PPP2R2B*. The second event is Myc phosphorylation, which is also inhibited by *PPP2R2B* re-expression in HCT116 cells. The other examined oncogenic signals, such as p-AKT, p-ERK, β-catenin and p-p38 were not affected by *PPP2R2B* re-expression (Figure4.11), which suggests the substrate specificity of *PPP2R2B* as a regulatory component of PP2A complex.

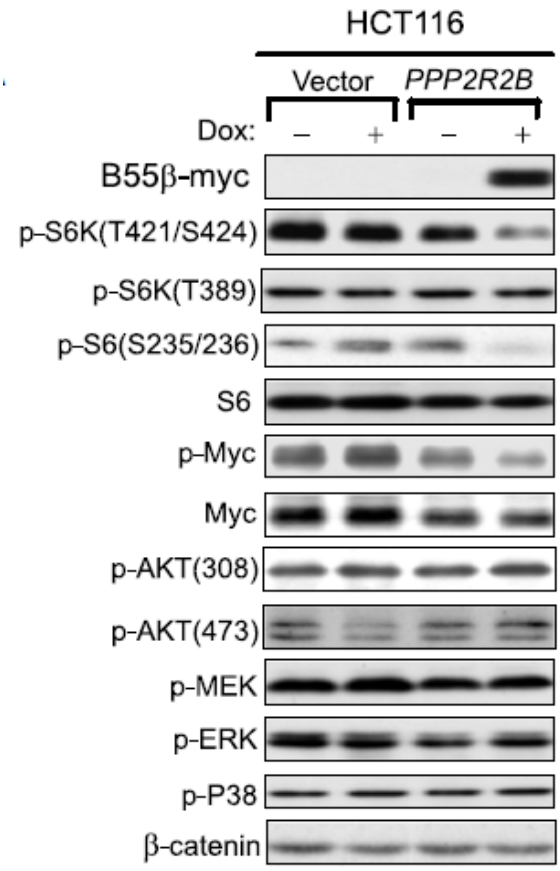


Figure 4.11 PP2A-PPP2R2B complex inhibits p70S6K and Myc phosphorylation

Ectopic expression of *PPP2R2B* in HCT116 inducible cells blocks p70S6K (T421/S424) and Myc phosphorylation.

Restoration of *PPP2R2B* complex activity abolishes p70S6K and Myc phosphorylation in a time dependent manner

The effects of *PPP2R2B* on p70S6K and c-Myc (thereafter referred to as Myc) were further confirmed in DLD1 cells in a time-course analysis (Figure 4.13A). The data showed that Dox induction of *PPP2R2B* expression resulted in a rapid dephosphorylation of Myc and p70S6K (T421/S424, but not T389) relative to total p70S6K and Myc protein levels, suggesting that these changes are the early effects of *PPP2R2B* and unlikely to be the secondary effect of growth inhibition. The decreased Myc phosphorylation eventually resulted in a lower level of total Myc protein after 48 hr of Dox treatment (Figure 4.13B), which is consistent with the previous finding that increased Myc phosphorylation at S62 correlates with Myc protein accumulation (Arnold and Sears, 2006; Junttila et al., 2007; Yeh et al., 2004). Thus, p70S6K and Myc are two downstream signals affected by *PPP2R2B*-PP2A complex, whereas no marked differences were observed in the expression levels nor phosphorylation status of other oncogenic signaling pathways known to be important in colorectal cancer.

Of note, *PPP2R2B* re-expression neither effected AKT T308 phosphorylation (Figure 4.11), which known to be targeted by PP2A/B55α or B56β complex in NIH 3T3 cells (Kuo et al., 2008; Padmanabhan et al., 2009), nor β-catenin phosphorylation is a target of PP2Aα in CRC (Su et al., 2008). In previous reports, SV40 small t antigen(ST)-mediated PP2A inhibition is associated with increased AKT S473 phosphorylation and the mTOR-mediated p70S6K T389 phosphorylation

in human mammary epithelial cell cells (Andrabi et al., 2007; Chen et al., 2005; Zhao et al., 2003), but these changes were not observed in this cellular context. These findings are consistent with the substrate specific functions of different PP2A/B subunits in various tissue specific contexts and show that *PPP2R2B*-associated PP2A complex is distinct from the other PP2A complexes by affecting different downstream substrates.

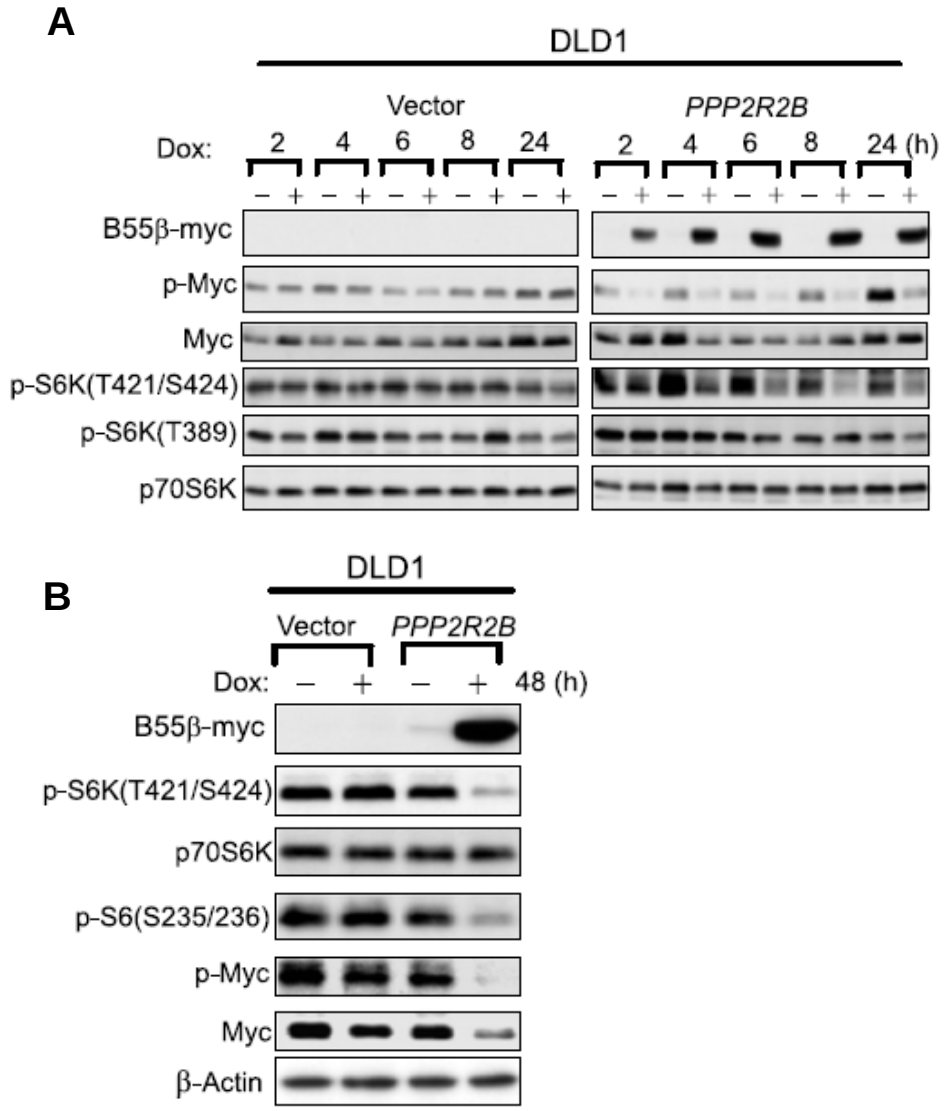


Figure 4.12 *PPP2R2B* re-expression blocks S6K and Myc phosphorylation, as well as Myc accumulation in DLD1 inducible cells

(A) Immunoblot analysis of DLD1-*PPP2R2B* or the vector control cells treated with or without Dox for the indicated length of times.
(B) Immunoblot analysis of DLD1-*PPP2R2B* or the vector control cells treated with or without Dox for 48 hr.

PPP2R2B binds to PP2A A and C subunit to form an active PP2A complex

Functional PP2A complex is composed of the structural A and catalytic C subunit, and a regulatory B subunit. *PPP2R2B* is a B regulatory subunit of PP2A, which forms an active PP2A complex with A and C subunits to mediate its tumor suppressor function. To demonstrate that re-expression of *PPP2R2B* leads to the formation of the active PP2A complex with other subunits, Co-immunoprecipitation assays was performed using Myc antibody to pull down the *PPP2R2B*-Myc fusion protein and its binding partners. Immunoblotting analysis of the immunoprecipitates shows that the ectopic expression of *PPP2R2B* co-immunoprecipitated with both PP2A structural (A) and catalytic (C) subunits to form a complex (Figure 4.13A). Furthermore, in an *in vitro* PP2A phosphorylation activity assay using a synthetic phosphothreonine peptide RRA(pT)VA as the substrate (Chen et al., 2004), the immunoprecipitates of *PPP2R2B* from Dox-treated DLD1-*PPP2R2B* cells clearly displayed increased PP2A phosphatase activity compared to untreated DLD1-*PPP2R2B* or Dox-treated vector control cells (Figure 4.13B), suggesting that *PPP2R2B* re-expression restored the previously decrease *PPP2R2B*-associated PP2A activity in CRC cells.

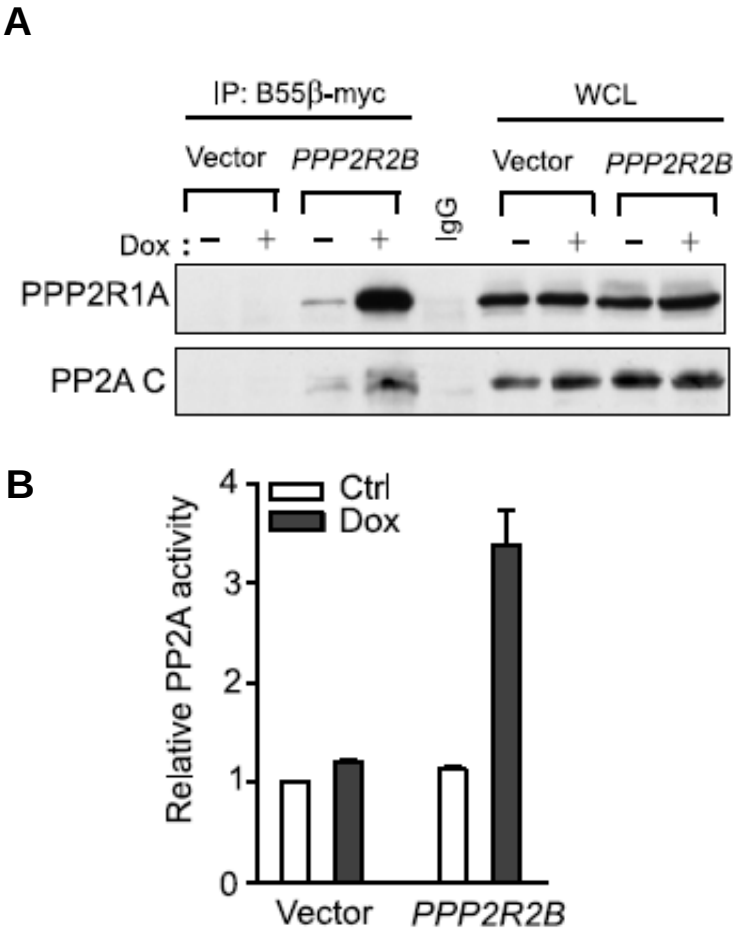


Figure 4.13 *PPP2R2B* binds to PP2A A and C subunits to form functional PP2A complex

(A) Coimmunoprecipitation of PP2A scaffolding A subunit and catalytic C subunit with *PPP2R2B*. DLD1-*PPP2R2B* or vector control cells were treated with Dox and *PPP2R2B* was immunoprecipitated with anti-Myc tag antibody. WCL, whole cell lysates.

(B) Serine/threonine phosphatase activity for DLD1-*PPP2R2B* or vector control cells. Protein phosphatase activity of the immunoprecipitates of *PPP2R2B*-Myc was measured in triplicates from three independent experiments.

PP2A activity is required for dephosphorylation of p70S6K and Myc by *PPP2R2B* re-expression

To confirm that dephosphorylation of p70S6K (T421/424) or Myc on *PPP2R2B* re-expression requires PP2A activity, we depleted the A subunit of PP2A complex by siRNA in DLD1-*PPP2R2B* cells and showed that this manipulation clearly prevented the dephosphorylation of Myc and p70S6K by *PPP2R2B* in PPP2R1A knockdown cells compared to siNC cells (Figure 4.14A). These experiments provided evidence to demonstrate a functional role of the *PPP2R2B*-PP2A complex in modulating p70S6K and Myc phosphorylation. In addition, we carried out co-immunoprecipitation to demonstrate that p70S6K and Myc are direct substrates of PP2A-*PPP2R2B*. The co-IP results showed that p70S6K was detected in PP2A-*PPP2R2B* immunoprecipitates, which indicated that p70S6K physically interact with *PPP2R2B* and direct target of *PPP2R2B*-associated PP2A complex (Figures 4.14B and 14C). However, we were unable to detect the physical interaction between Myc with *PPP2R2B*. Although another PP2A subunit B56α has been previously shown to interact and dephosphorylate Myc in HEK293 cells (Arnold and Sears, 2006; Junttila et al., 2007), *PPP2R2B*-PP2A complex may modulate Myc phosphorylation indirectly in CRC cells. Taken together, these findings suggested that PP2A activity is required for p70S6K and Myc dephosphorylation either through direct or indirect modulation.

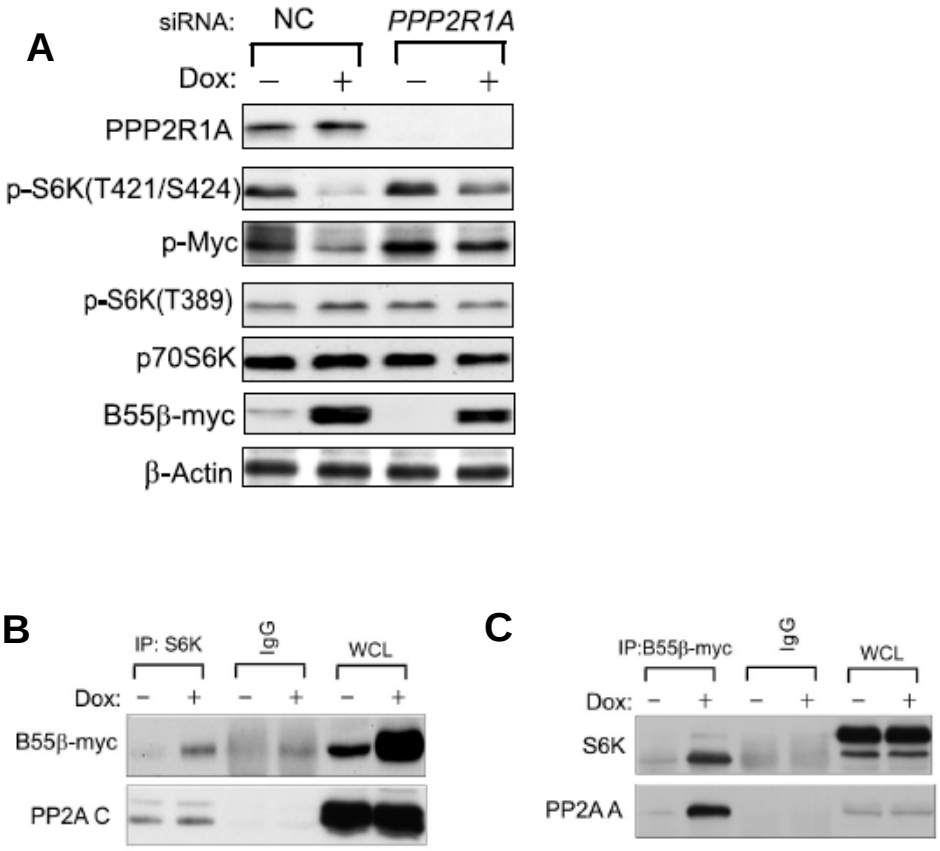


Figure 4.14 PP2A activity is required for dephosphorylation of p70S6K and Myc by PPP2R2B re-expression

(A) Immunoblot analysis of DLD1-*PPP2R2B* cells for Myc, p-Myc (S62), and p70S6K, treated PPP2R1A siRNA or a negative control siRNA, with or without Dox treatment.

(B) DLD1-*PPP2R2B* cells were treated with Dox for 8 h and cell extracts were subjected to immunoprecipitation with S6K antibody and interaction of S6K with B55ß and PP2A C subunit were studied by immunoblotting of the immunoprecipitates.

(C) DLD1-*PPP2R2B* cells were treated as (A) and cell lysate were immunoprecipitated with Myc-tagged antibody and the interaction of B55ß with S6K and PP2A A subunit were studied by Western blotting of the immunoprecipitates.

Downregulation of Myc but not p70S6K inhibit cell growth in CRC

To evaluated the functional significance of Myc and p70S6K downregulation in CRC, siRNA targeting p70S6K or Myc were used to examine the functions in DLD1 and HCT116 cells. Cell proliferation were measured by CellTiter-Glo® Luminescent Cell Viability Assay Kit after siRNA treatment. The data showed that Myc knockdown strongly suppressed DLD1 cell viability, whereas p70S6K knockdown did not yield a significant effect in both HCT116 and DLD1 cells(Figure 4.15A). Moreover, *PPP2R2B* re-expression did not induce further growth inhibition when Myc was depleted in the cells (Figure 4.15B). Therefore, this data indicate a functional contribution of Myc inhibition to the growth inhibitory effect of B55β, which is consistent with the established role of Myc in colorectal tumorigenesis (Korinek et al., 1997; Morin et al., 1997; Sansom et al., 2007).

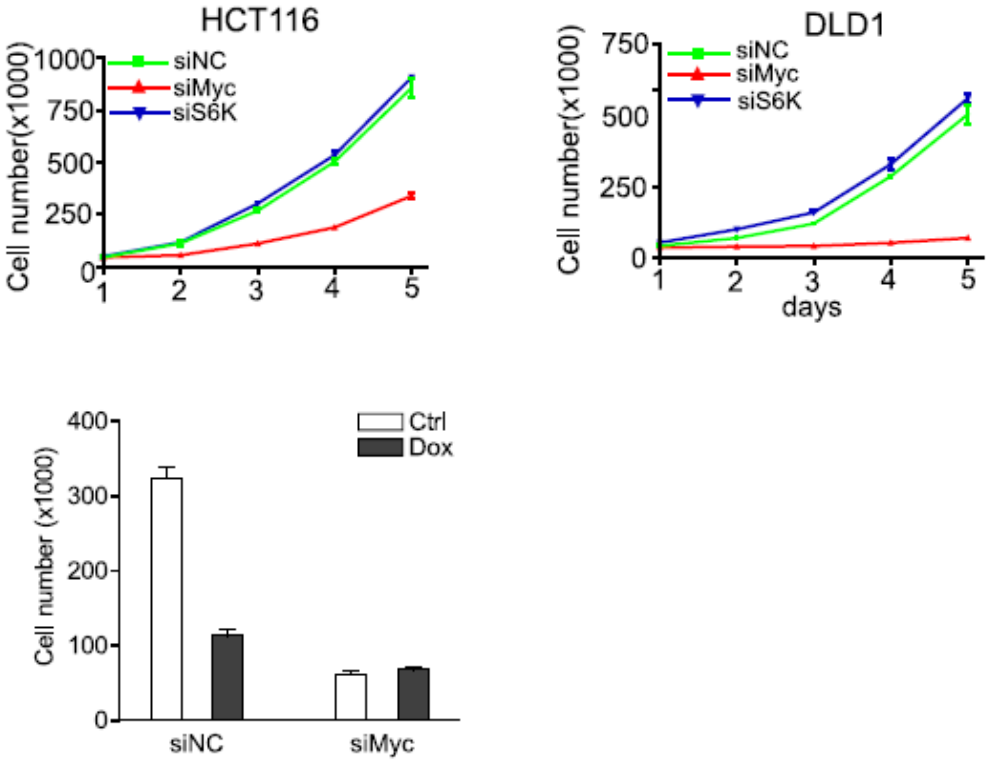


Figure 4.15 Myc knockdown blocks cell viability in CRC

(A) Growth curve of HCT116 and DLD1 cells treated with Myc, p70S6K siRNA, or negative control siRNA for indicated number of days.
(B) Growth inhibition of DLD1-*PPP2R2B* cells induced by Myc knockdown, *PPP2R2B* expression or both.

4.3 Discussion

In this chapter, we have demonstrated that *PPP2R2B* which encodes for the PP2A regulatory B55β subunit, is epigenetically inactivated by DNA hypermethylation in the promoter in colorectal cancer. The functional effects of re-expression of *PPP2R2B* in CRC reverses the cancer phenotypes, which include senescence, decreased cell proliferation, and xenograft tumor growth inhibition. In contrast, *PPP2R2B* knockdown promotes cell transformation in immortalized epithelial cells. Despite current evidence has established that loss of PP2A is required for malignant transformation (Chen et al., 2004; Janssens et al., 2005; Zhao et al., 2003), only a few reports thus far have presented potential mechanisms for the inhibition of PP2A activity in spontaneously developing human tumors (Junttila et al., 2007; Neviani et al., 2005; Sablina et al., 2007). In this regard, low-frequency genetic alterations of PP2A subunits have been found in human cancers (Calin et al., 2000; Wang et al., 1998), which make it difficult to reconcile PP2A's role as a *bona fide* tumor suppressor with little evidence showing the profound and widespread alterations of PP2A subunits in human cancers. In addition, overexpression of PP2A inhibitor proteins, such as CIP2A and SET, were recently reported to promote malignant growth through inhibiting PP2A tumor suppressor activity. Considering this evidence, it is clear that the identification of *PPP2R2B* with tumor suppressor function is important and expands understanding of the underlying mechanisms how PP2A works as tumor suppressor in CRC. In addition to colorectal cancer, *PPP2R2B* may also be downregulated in other cancers such as bladder, brain and esophagus

carcinoma, as suggested by Oncomine database. Thus, considering the low frequency of PP2A mutations among various subunits (<15%), the epigenetic mechanism leading to PP2A inactivation may play a more significant role in the development of human cancer like CRC.

Due to the substrate diversity of PP2A regulatory subunits, it is not surprising to observe numerous oncogenic signaling pathways affected in various tissues or cellular contexts. We hypothesize that the loss of *PPP2R2B* associated specific PP2A complex may lead to the disruption of certain oncogenic signaling pathways required for CRC cell survival and proliferation. Thus, this study explored the role of *PPP2R2B* in regulating of these oncogenic pathways in CRC. The mTOR downstream effector and oncogenic protein Myc were identified as direct or indirect targets of *PPP2R2B*. Among these substrates, Myc was identified as a crucial downstream target of *PPP2R2B* and inactivation of Myc by PP2A-*PPP2R2B* was consistent with the strong growth inhibition effect of *PPP2R2B* on CRC cells. This is in agreement with a crucial role of Myc in CRC tumorigenesis (Sansom et al., 2007). Although *PPP2R2B* has no discernable effect on Wnt pathway in CRC cells, it regulates Myc phosphorylation and protein accumulation. Thus its effect on Myc is consistent with the strong growth inhibition effect of *PPP2R2B* on CRC cells that are addicted to Wnt pathway for growth advantage. It is well established that Myc is one of substrate targets for PP2A (Arnold and Sears, 2006; Junttila et al., 2007; Yeh et al., 2004); ectopic expression of SV40 small T or PP2A inhibitor protein CIP2A regulate myc stablitiy through association and phosphorylation of Myc. In this study, we show that

Myc is not the direct substrate of *PPP2R2B*-associated PP2A complex, which indicate that loss of *PPP2R2B* may activiate unknown kinase activity, which lead to Myc phosphorylation and accumulation in human malignancies. Thus, further investigation of the specific substrates targeted by *PPP2R2B* in PP2A complex may provide an alterative signaling pathway leading to deregulation of Myc in the future study.

5 Chapter V : *PPP2R2B* controls PDK1-directed Myc signaling and modulates rapamycin sensitivity in colon cancer

5.1 Introduction

The phosphatidylinositol 3-kinase (PI3K)-AKT pathway plays a central role in cancer development and it is one of the most frequently deregulated signaling pathways in human cancers (Cully et al., 2006; Vivanco and Sawyers, 2002). Genetic aberrations in this pathway, such as activating mutations of PIK3CA or inactivation of PTEN, are identified virtually in all epithelial tumors (Li et al., 1997; Samuels et al., 2004). Activation of this pathway in cancer suggests that PI3K, AKT, and other components of this pathway may be attractive targets for cancer therapy. One of the PP2A regulated cancer signaling pathways is mTOR (Mammalian Target Of Rapamycin) pathway which is a key component of PI3K-AKT pathway that many cancer cells are coferred with a growth advantage following its constitutive activation (Guertin and Sabatini, 2007; Sabatini, 2006).

In recent years, inhibitors targeting mTOR pathway are under active clinical development as anti-cancer therapeutics. Although small molecule mTORC inhibitors, such as rapamycin and its analogs have shown promising rationale for its use in cancer therapy (Guertin and Sabatini, 2009; Hudes et al., 2007), these inhibitors have limited efficacies and the clinical outcomes are often unpredictable. Thus, rapamycin resistance has emerged as a major challenge for clinical application. The well-documented mechanisms of rapamycin resistance are often linked to its negative feedback loops, which partially resulted in hyperactivation of PI3K/AKT and MAPK/ERK signaling in rapamycin-treated cells (Carracedo et al., 2008a; Carracedo et al., 2008b; Sarbassov et al., 2006). However, the mechanisms

underlying the development of rapamycin resistance remain elusive. Therefore, a deeper understanding of cancer cells acquired rapamycin resistance and the identification of biomarkers that could help to predict therapeutic responses are pivotal to the success of the clinical application of mTOR inhibitors (Mao et al., 2008; Scott et al., 2009; Thomas et al., 2006; Yu et al., 2011).

In the Chapter four, we described a subunit of the PP2A holoenzyme, *PPP2R2B*, which was epigenetically inactivated in human colorectal cancer. Gain- and loss-of-function analyses revealed that *PPP2R2B*-associated PP2A complex works as a tumor suppressor in CRC by negatively inhibiting Myc phosphorylation and mTOR downstream effector p70-S6K phosphorylation leading to the phosphorylation of S6 ribosomal protein. The connection between *PPP2R2B* and mTOR/S6K/S6 signaling pathway inspired us to investigate the hypothesis that *PPP2R2B*-associated PP2A complex may be implicated in the cellular sensitivity of mTOR inhibitor rapamycin in CRC. In this chapter, rapamycin was found to induce strong Myc phosphorylation which causes aquired resistance in CRC. Restoration of the *PPP2R2B*-associated PP2A complex inhibits rapamycin-induced Myc phosphorylation and enhances the sensitivity of CRC to rapamycin. In contrast to the previously described rapamycin-induced AKT phosphorylation, induction of Myc phosphorylation by rapamycin in our system is depdent on PDK1 instead of PI3K/AKT feedback pathway. Hence, we identified a *PPP2R2B*-PP2A tumor suppressor mechanism controlling PDK1 and Myc phosphorylation and propose that a epigenetic defect in this mechanism in cancer might facilitate tumor cells to

acquire resistance to mTOR-based cancer therapy.

5.2 Results

5.2.1 *PPP2R2B* re-expression sensitizes mTOR inhibitor rapamycin

PPP2R2B re-expression in CRC sensitizes mTOR inhibitor rapamycin in vitro

The mTOR kinase inhibitor rapamycin has a sporadic anti-cancer activity and its effect on mTOR downstream substrate p70S6K is often used as a surrogate marker to evaluate rapamycin response (Sawyers, 2008). The plausible connection between *PPP2R2B* and p70S6K signaling leads us to investigate the possibility that *PPP2R2B* expression status may affect the cellular sensitivity to rapamycin. We thus compared the effect of rapamycin on cell viability of DLD1-*PPP2R2B* cells in the presence or absence of Dox. DLD1-*PPP2R2B* cells were treated with 10nM mTOR inhibitor rapamycin following 10ng/ml Dox treatment for 7 days. Cell viability was measured using CellTiter-Glo™ Luminescent Cell Viability Assay Kit every other day. We treated DLD1-vector cells in the same manner as a negative control. The proliferation assay showed that rapamycin-induced growth inhibition was much more effective when *PPP2R2B* was re-expressed following Dox treatment in DLD1-*PPP2R2B* cells, but not in the control cells (Figure 5.1A). Moreover, colony formation assay also indicated that *PPP2R2B* re-expression increases the sensitivity of rapamycin treatment in CRC cells when we treated with 10nM rapamycin in the absence or

presence of Dox in DLD1-vector and DLD1-*PPP2R2B* cells for 14 days (Figure 5.1B). In addition, cell cycle analysis by flow cytometry was carried out in an attempt to understand the mechanisms involved in the synergy effects of *PPP2R2B* and rapamycin in the suppression of cell growth and cell proliferation. DLD1 inducible cells were treated with 10nM rapamycin in the presence or absence of Dox for 48 hr for flow cytometer analysis. The FACS results showed that Dox induction of *PPP2R2B* expression resulted in cell cycle G2 arrest, which was further augmented with the addition of rapamycin, indicating that *PPP2R2B* and rapamycin synergistically induced cell cycle arrest in G2/M phase (Figure 5.2). In generally, these results indicated that the restoration of *PPP2R2B* increases the sensitivity of CRC to rapamycin to inhibit cell viability and proliferation through cell cycle arrest in G2/M phase.

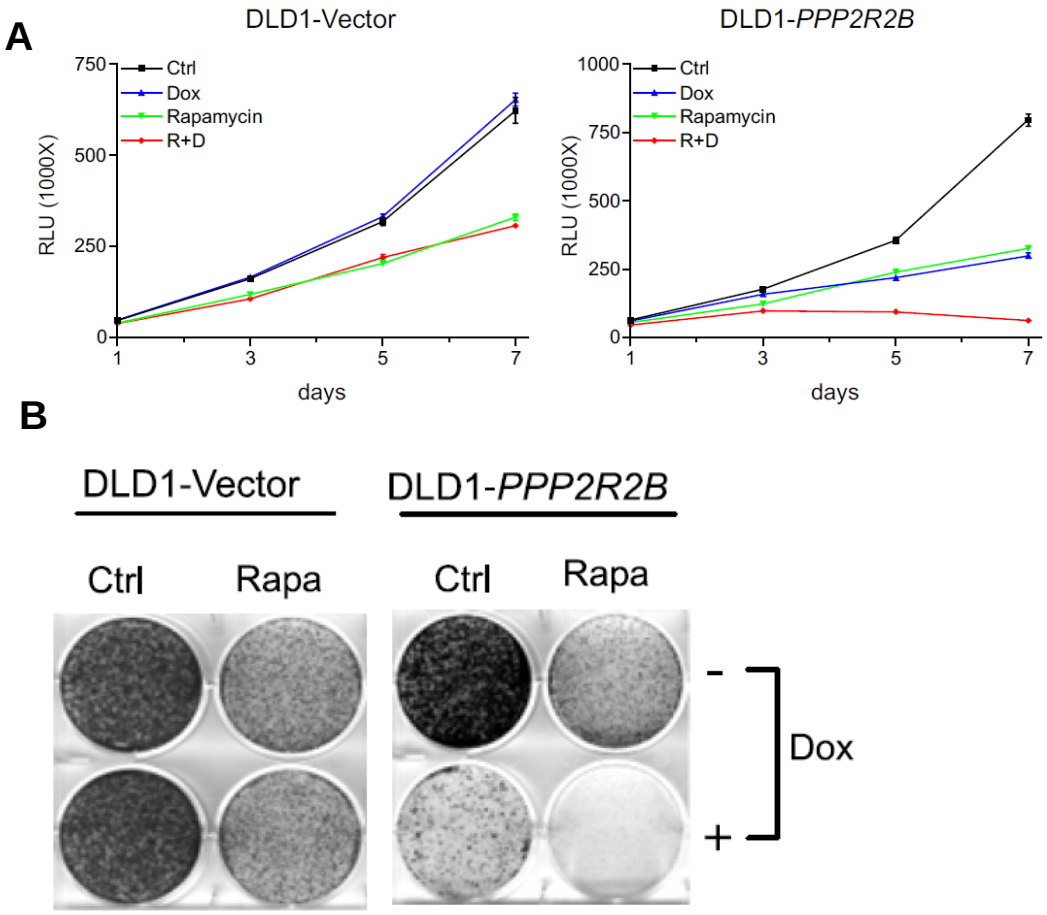


Figure 5.1 PPP2R2B re-expression and rapamycin treatment synergistically inhibits cell growth and cell proliferation.

(A) Proliferation of DLD1-vector and DLD1-*PPP2R2B* cells treated with 10 ng/ml Dox or 10 nM rapamycin or both (R+D) for indicated days. Error bar shows the mean ± SD of triplicates. RLU represents relative light unit.
(B) Dense foci formation on a monolayer of DLD1-vector or DLD1-*PPP2R2B* cells treated with 10 nM rapamycin, with or without 10 ng/ml Dox treatment for 14 days.

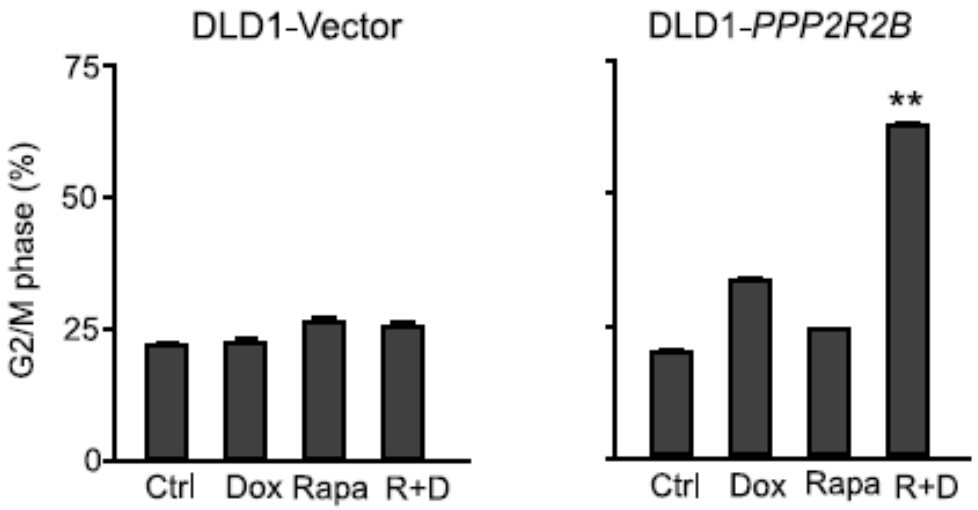


Figure 5.2 PPP2R2B re-expression and rapamycin treatment synergistically induced cell cycle arrest in G2/M phase.

Cell cycle G2/M arrest in DLD1-vector or DLD1-*PPP2R2B* cells treated with 1.0μg/ml Dox or 10nM rapamycin or both for 48 hr. Error bar shows the mean ± SD of three independent experiments. ** $p<0.001$

PPP2R2B re-expression in CRC sensitizes cells to rapamycin in vivo

To verify the *in vitro* results *in vivo*, we studied the effects of Dox and rapamycin on xenograft tumor growth in nude mice using DLD1-*PPP2R2B* cells. While single treatment with rapamycin or Dox resulted in modest attenuation of the tumor growth; the combination gave rise to a strong anti-tumor growth effect in the DLD1-*PPP2R2B* cells as compared to the control cells (Figure 5.3). The results obtained from both in vitro and in vivo studies collectively indicated that the *PPP2R2B* re-expression in CRC cells led to improved therapeutic effect of rapamycin. Thus, the data suggested that epigenetic loss of *PPP2R2B* may be a molecular event affecting the sensitivity of CRC to mTOR inhibitors.

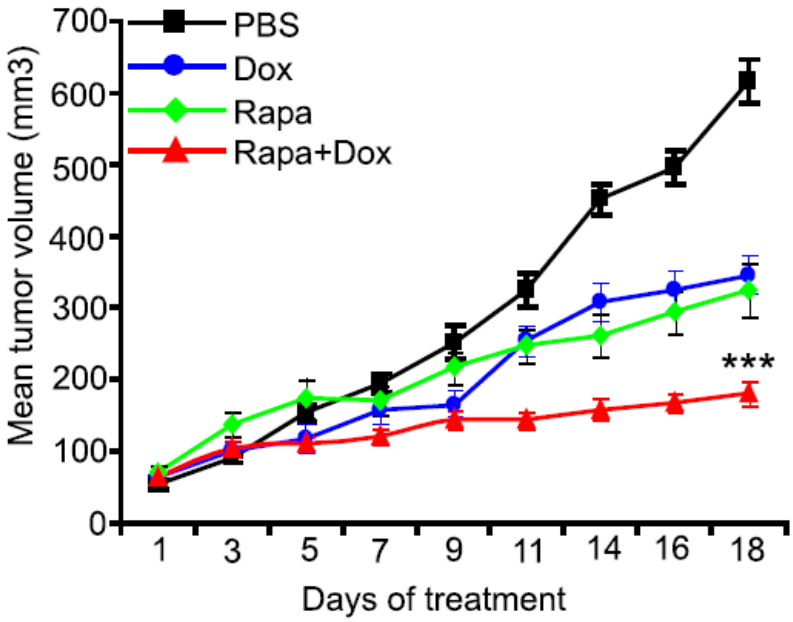


Figure 5.3 Xenograft tumor growth of DLD1-PPP2R2B cells in nude mice

Xenograft tumor growth of DLD1-*PPP2R2B* cells in nude mice treated with Dox at 100 mg/kg, or rapamycin at 4 mg/kg or both, every other day. Error bars represent ± SEM (n = 8 per group). ***$p < 0.01$(independent t test).

5.2.2 Rapamycin induces Myc phosphorylation and protein accumulation in CRC cells, which is overridden by *PPP2R2B* re-expression

Rapamycin induced Myc phosphorylation is blocked by *PPP2R2B* re-expression

A feedback mechanism leading to PI3K activation and AKT S473 phosphorylation in a mTORC2-depenent manner has been linked to rapamycin resistance in cancer (O'Reilly et al., 2006; Sarbassov et al., 2006). Indeed, rapamycin treatment of CRC cells resulted in the induction of AKT S473 phosphorylation, but this phosphorylation seemed to be unaffected by *PPP2R2B* re-expression (Figure 5.4A). In contrast, both p70S6K and S6 phosphorylation were effectively abolished by rapamycin treatment in both vector control and *PPP2R2B* expressing cells (Figure 5.4A). Thus we excluded the possibility that *PPP2R2B*-induced rapamycin sensitization is associated with AKT S473 or p70S6K. On the contrary, and intriguingly, we found that rapamycin treatment resulted in a strong induction of Myc phosphorylation and protein accumulation. This effect on Myc protein was not due to increased Myc mRNA as detected by real-time qPCR (Figure 5.4B) and was nearly completely abolished upon *PPP2R2B* re-expression (Figure 5.4A). To further evaluate the effect of rapamycin in Myc phosphorylation in CRC, a time course treatment of rapamycin was performed in DLD1 cells. Immunoblotting analysis indicates that the Myc response occurred as early as 4 hours, in parallel with the induction of AKT S473 phosphorylation, revealing an additional feedback event in response to rapamycin (Figure 5.4C).

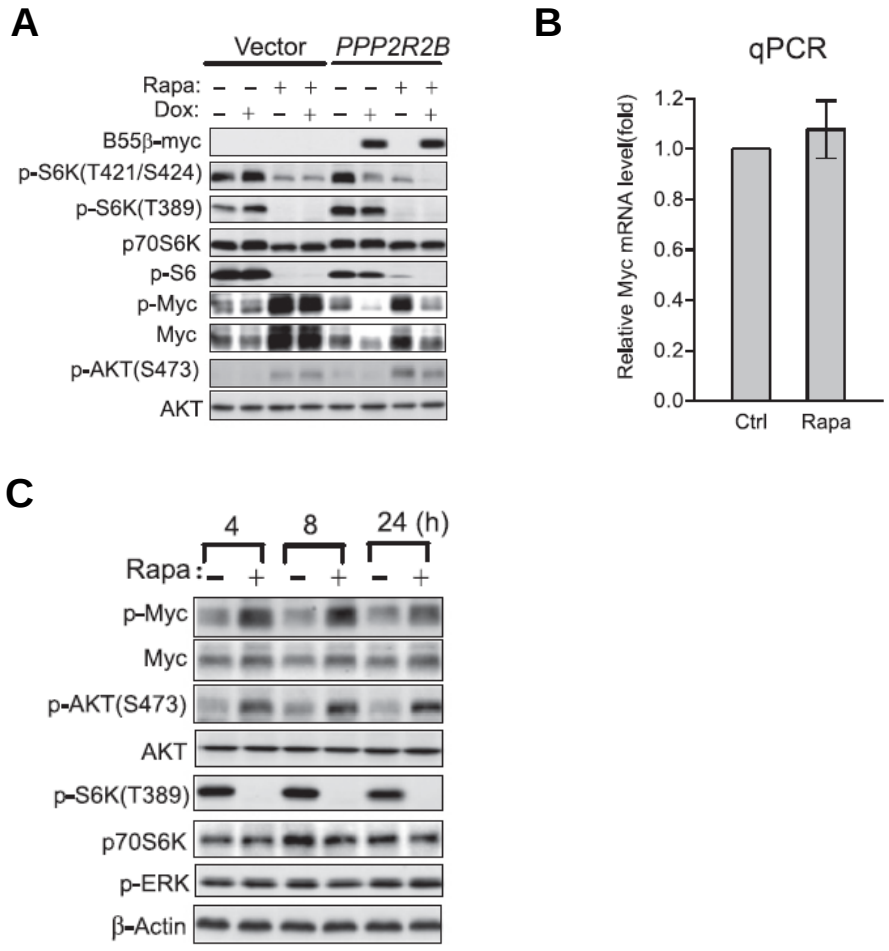


Figure 5.4 Rapamycin Induced Myc Phosphorylation and protein accumulation in CRC cells

(A) DLD1-*PPP2R2B* or DLD1 vector control cells were treated with 10 nM rapamycin in the presence or absence of Dox for 48 hr. The immunoblotting analysis shows that rapamycin induces Myc S62 phosphorylation, which is abrogated on *PPP2R2B* expression.

(B) TaqMan assay of Myc mRNA change in response to rapamycin treatment. Bar respresents the mean ± SD of triplicates.

(C) Immunoblotting analysis of indicated proteins in DLD1 cells treated with 10 nM rapamycin at the indicated time points.

Rapamycin induces Myc phosphorylation through mTORC1 inhibition, but not mTORC2 inhibition

Rapamycin-induced AKT(S473) phosphorylation is mediated by a feedback loop through mTORC2- and PIK3CA-dependent manner, which linked to rapamycin resistance (Sarbassov et al., 2006). To investigate whether induction of Myc by rapamycin is the same pathway as AKT activation, retroviral shRNA vectors targeting mTORC1 and mTORC2 components were introduced into DLD1 cells and were subsequently harvested for immunoblotting analysis. As shown in figure 5.5, the rapamycin-induced Myc phosphorylation and accumulation was indeed the result of mTORC1 inhibition, as knockdown of raptor, an essential component of mTORC1, but not mTORC2 component rictor, resulted in a similar induction of Myc phosphorylation. Given the importance of Myc in CRC tumorigenesis, this observation immediately suggests a possible mechanism underlying the *PPP2R2B*-mediated sensitization to rapamycin.

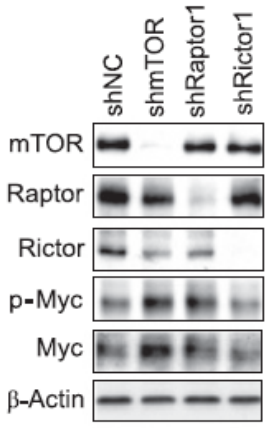


Figure 5.5 Rapamycin induces Myc phosphorylation through mTORC1 inhibition

Immunoblotting analysis of Myc in DLD1 cells treated with shRNAs targeting mTOR, raptor, or rictor.

Lack of *PPP2R2B* expression correlates with rapamycin resistance and Myc response

To substantiate the association of *PPP2R2B* expression with Myc response and rapamycin sensitivity, we compared the HCT116 cells with DNMTs-deficient HCT116 (DKO) cells in which *PPP2R2B* becomes re-expressed as a result of promoter demethylation. As in DLD1 cells, Myc phosphorylation was strongly induced by rapamycin in HCT116 cells, whereas in DKO cells, Myc was expressed in a low basal level, and did not respond to rapamycin (Figure 5.6A). Notebly, AKT Serine 473 phosphorylation was similarly induced by rapamycin in both cell lines, regardless of *PPP2R2B* expression status (Figure 5.6A). Accordingly, DKO cells which displayed no Myc response were more sensitive to rapamycin treatment as compared to the parental HCT116 cells (Figure 5.6B). Taken together, the effect of *PPP2R2B* on Myc highly correlated with the rapamycin response and support a role of *PPP2R2B* in regulating Myc phosphorylation and thus rapamycin sensitivity. Moreover, corresponding to the consistent silencing of *PPP2R2B* in CRC, all the CRC cell lines we have examined exhibited a marked induction of Myc phosphorylation upon rapamycin treatment (Figure 5.6C). In agreement with the Myc induction, all these CRC cell lines were in general resistant to rapamycin, showing the growth inhibition for less than 10% after 5 days treatment with 10 nM rapamycin (Figure 5.8B), a concentration that often results in strong growth inhibition in other cancer cell lines (Figure 5.8B). This indicated that the lack of *PPP2R2B* in CRC cells is associated with the rapamycin resistance.

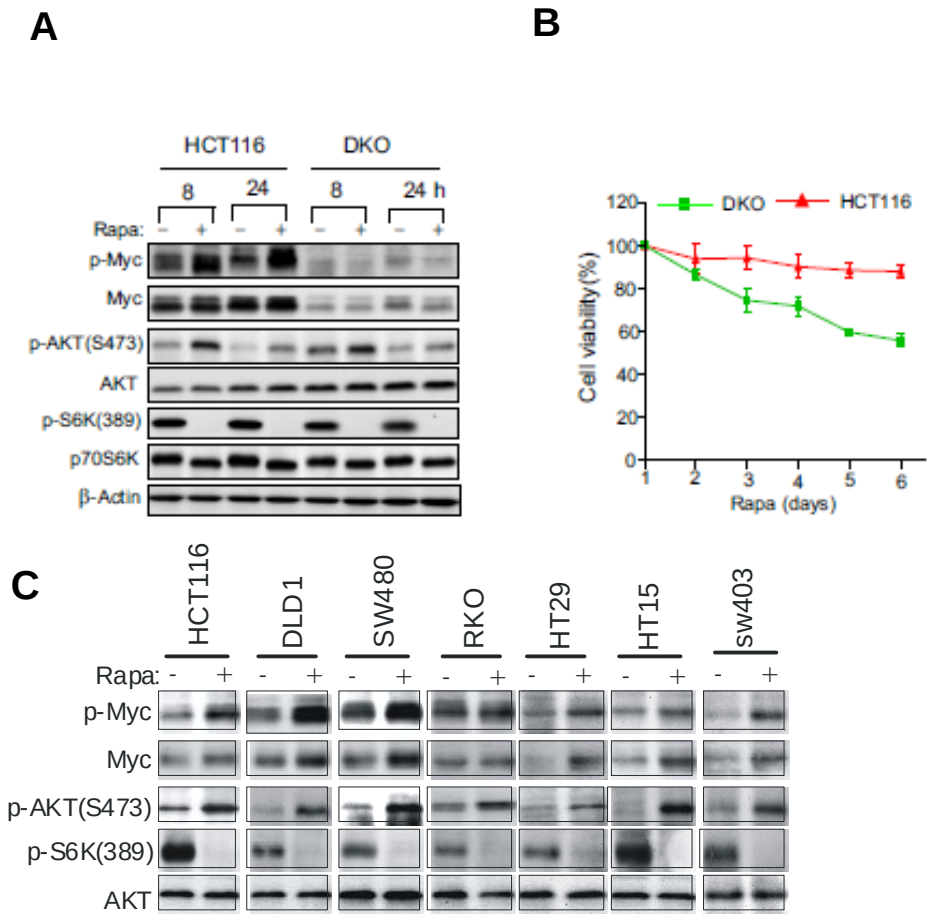


Figure 5.6 Lack of PPP2R2B expression correlates with rapamycin resistance and Myc response

(A) Immunoblot analysis of Myc, AKT and p70S6K in HCT116 and DKO cells treated with10 nM rapamycin for the indicated times

(B) Rapamycin induced growth inhibition in HCT116 and DKO cells.

(C) Immunoblotting analysis of indicated proteins in CRC cell lines treated with or without rapamycin.

(D) Growth inhibition induced by rapamycin in CRC cell lines assessed by CellTiter-Glo Luminescent Cell Viability Assay and normalized to untreated cells. Bar shows the mean ± SD of triplicates.

PPP2R2B expression determines Myc induction and rapamycin sensitivity

In contrast to colon cancer, the Oncomine database revealed increased *PPP2R2B* expression in several other human malignancies, including renal, liver, and ovarian cancers (Figure 5.7A). Real-time Taqman assay validated the *PPP2R2B* expression in a series of cell lines derived from above tumors, including HepG2 and HepB3 cells form hepotoma; 786-O and Caki2 cells from renal carcinoma; OVCAR3, OVCAR5, and SK-OV-3 cells from ovarian carcinoma; as well as U2OS cells from osteosarcoma, as opposed to the silenced expression of *PPP2R2B* in CRC lines (Figure 5.7B). To check whether the *PPP2R2B* expression determines Myc induction and rapamycin sensitivity, all cancer cells were treated with 10nM rapamycin for 48 hr and harvested for immunoblotting analysis. As shown in figure 5.8A, rapamycin consistently induces the AKT S473 phosphorylation and completely suppresses the S6K T389 phosphorylation. Of significant notice, none of these *PPP2R2B*-expressing cancer cell lines showed an induction of Myc phosphorylation in response to rapamycin, despite a consistent induction of AKT S473 phosphorylation in all cases (Figure5.8A). Moreover, these cell lines were much more sensitive to rapamycin in general, as compared to CRC cell lines (Figure 5.8B). The overall results have provided evidence to show: 1) the *PPP2R2B* silence or expression in cancer cells correlates with the sensitivity or resistance to rapamycin; 2) This correlation is associated with at least in part the ability of rapamycin to induce Myc phosphorylation.

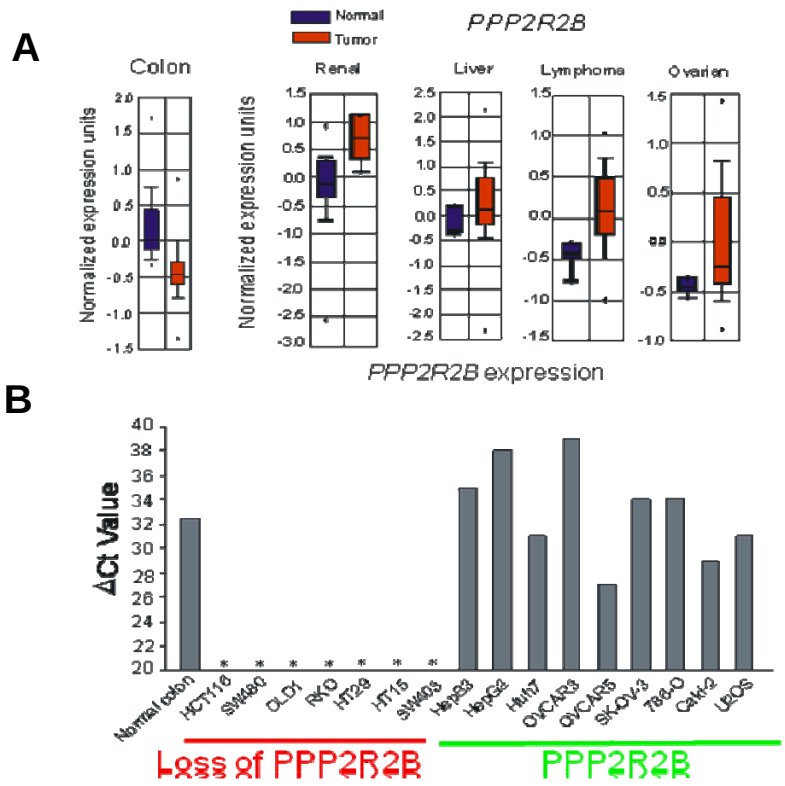


Figure 5.7 PPP2R2B is not downregulated in renal, liver, lymphoma and ovarian cancer cells

(A) Box-plots showing the differential expression of *PPP2R2B* mRNA in renal (Boer etal., 2001), liver (Chen et al., 2002), lymphoma (Alizadeh et al., 2000), ovarian cancer (Hendrix et al., 2006) and corresponding normal tissues obtained from ONCOMINE database. The y-axis represents normalized expression and the *P*-Values for correlation are shown below.
(B) Taqman assay of *PPP2R2B* mRNA in indicated cancer cell lines and normal colon mucosa. CT values were shown, * means undetectable value.

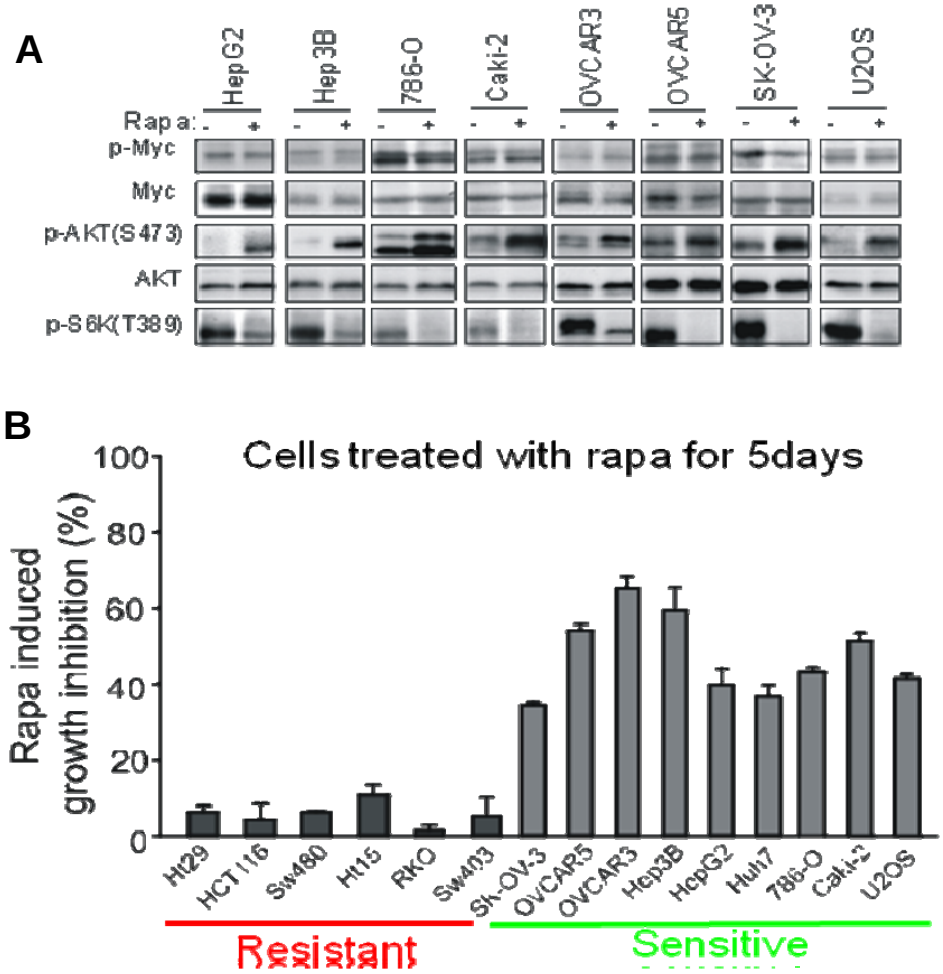


Figure 5.8 Expression of PPP2R2B in cancer cells correlates with Myc induction and rapamycin response.

(A) Immunoblot analysis of indicated proteins in non-CRC cell lines that express *PPP2R2B*, treated with or without rapamycin.
(B) Growth inhibition induced by rapamycin in CRC cell lines and non-CRC cancer cell lines as assessed by CellTiter-Glo Luminescent Cell Viability Assay and normalized to untreated cells. Cell were treated with 10nM repaying for 5 days. Bar shows the mean ± SD of triplicates.

5.2.3 Rapamycin-induced Myc phosphorylation is PDK1-dependent, but *PIK3CA*-AKT independent.

Rapamycin induces Myc phosphorylation through PIK3CA-AKT independent manner.

mTORC2-dependent AKT S473 activation depends on receptor tyrosine kinase signaling upon growth factor stimulation (Guertin and Sabatini, 2007; Sekulic et al., 2000), and *PIK3CA* (encoding p110α) is required for this process (Jia et al., 2008; Knight et al., 2006; Zhao et al., 2006). To investigate the relationship between *PIK3CA* and Myc induction, we cultured DLD1 cells with serum starvation medium (DMEM with 0.25% FBS) for 48 hr to eliminate the effect of growth factor in the cell culture. The serum starved cells were treated with 10nM rapamycin at different time courses and harvested for immunoblotting analysis. The results showed that serum starvation abolished rapamycin-induced AKT S473 phosphorylation, which was dependent on growth factor stimulation, and had no significant effect on rapamycin-induced Myc phosphorylation (Figure 5.9A). these data indicate that rapamycin–induced Myc phosphorylation is independent to PIK3CA. To further confirm this finding, we used PIK-103, a dual *PIK3CA* and mTORC1 inhibitor (Fan et al., 2006), to treat DLD1 cells and the immunoblotting analysis results showed that PIK-103 reduced AKT S473 phosphorylation, but enhanced Myc phosphorylation (Figure 5.9B). These findings suggest that rapamycin induces Myc phosphorylation through a distinct mechanism that does not depend on *PIK3CA*.

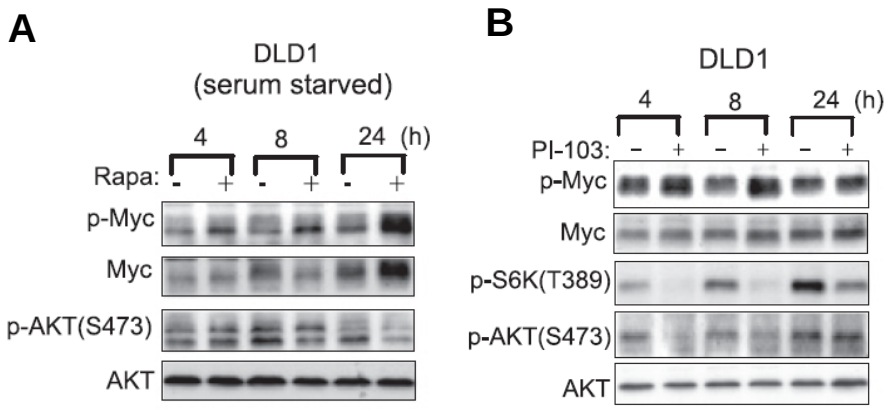


Figure 5.9 Rapamycin induces Myc phosphorylation through PIK3CA-AKT independent manner.

(A) Immunoblotting analysis of DLD1 cells for AKT and Myc. DLD1 cells were serum starved for 48 hr and followed by treatment with 10 nM rapamycin for the indicated time points.

(B) Immunoblotting analysis of DLD1 cells for AKT and Myc in response to a dual mTORC1 and *PIK3CA* inhibitor PI-103 (0.5 μM) for the indicated times.

Rapamycin-Induced Myc Phosphorylation Requires PDK1 but not PIK3CA-AKT pathway

To further elucidate the upstream signals leading to rapamycin-induced Myc phosphorylation, we knocked down several major components in the PI3K and mTOR pathway using siRNAs targeting *PIK3CA*, *PIK3CB*, PDK1 and AKT1. siRNA transfected cells were treated with 10nM rapamycin for 48 hr and cells were harvested for immunoblotting analysis. The western blot results showed that PDK1 knockdown effectively inhibited rapamycin-induced Myc phosphorylation, while knockdown of *PIK3CA*, *PIK3CB*, or *AKT*1 had no such an effect (Figure 5.10A). Further experiments with two additional PDK1 siRNAs and a PDK1 shRNA in DLD1 and SW480 cells confirmed its effect on Myc phosphorylation (Figure 5.10B and 10C); but of notice, PDK1 knockdown had no discernable effect on AKT-S473 phosphorylation, which however can be clearly abolished by *PIK3CA* knockdown (Figure 5.10A).

To substantiate this finding, we made use of specific small molecule inhibitor of PDK1, BX912 (Feldman et al., 2005) and a specific *PIK3CA* inhibitor PIK90 (Fan et al., 2006; Knight et al., 2006). SW480 cells were treated with BX912 or PIK90 in the presence or absence of rapamycin for 48 hr and harvested for immunoblotting analysis. Figure 5.10D indicated that BX912 treatment abolished rapamycin-induced Myc phosphorylation as consistent with PDK1 knockdown, but had not much effect on AKT S473. In contrast, PIK90 was unable to inhibit rapamycin-induced Myc phosphorylation; yet it can effectively abolished the AKT S473 phosphorylation

(Figure5.10D). Taken together, these results provided concrete evidence to show that rapamycin induces a PDK1-dependent Myc phosphorylation, in parallel to *PIK3CA*-sensitive AKT S473 phosphorylation.

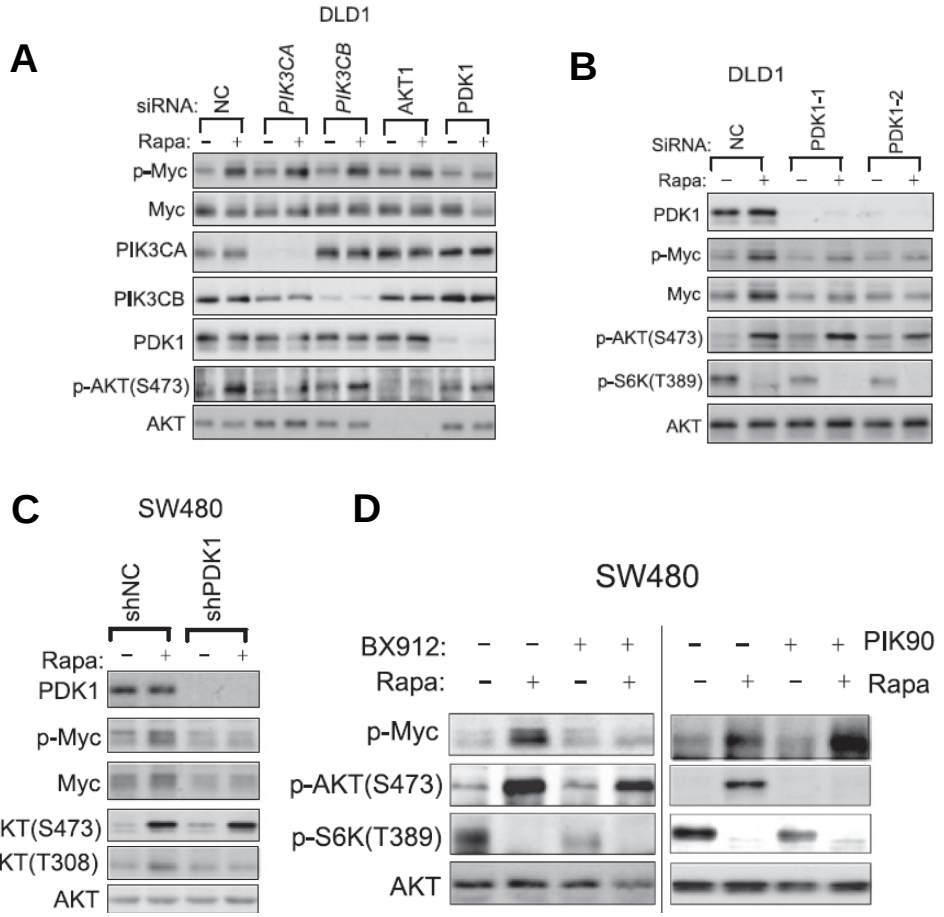


Figure 5.10 Rapamycin induced Myc phosphorylation requires PDK1 but not PIK3CA-AKT pathway.

(A) Immunoblot analysis of DLD1 cells for Myc, AKT, PI3K, or PDK1. Cells were transfected with siRNAs targeting the indicated genes or a negative control siRNA for 48 hr, followed by 10 nM rapamycin treatment for 24 hr.

(B) Immunoblot analysis of PDK1, Myc and AKT in DLD1 cells transfected with two different PDK1 siRNAs or a negative control siRNA and then treated with 10 nM rapamycin for 24 hr.

(C) Immunoblot analysis of PDK1, Myc, and AKT in SW480 cells infected with a retroviral PDK1 shRNA.

(D) Immunoblot analysis of Myc, AKT, and S6K in DLD1 cells treated with PDK1 inhibitor BX912 (2.5 mM), or p110a inhibitor PIK90 (5 mM), rapamycin (10 nM) or indicated combinations for 48 hr.

Ectopic expression of PDK1 leads to exogenous and endogenous Myc phosphorylation and Myc accumulation

PDK1 is the master regulator of AGC kinase members, including AKT, p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK) and protein kinase C (PKC) family members, which play crucial roles in regulating physiological processes such as metabolism, growth, proliferation and survival (Mora et al., 2004). In human cancers, elevation of PIP3 owing to loss of PTEN or gain of PIK3CA activity may cause constitutive activation of PDK1. In addition, gene amplification or altered phosphorylation can also contribute to excessive PDK1 activity in human malignancies such as breast and colon cancers (Lin et al., 2005; Maurer et al., 2009). To identify the relationship between PDK1 and Myc, we investigated whether ectopic expression of PDK1 can lead to Myc phosphorylation. Cotransfection of PDK1 and Myc in 293T cells resulted in a huge induction of Myc phosphorylation and protein accumulation (Figure 5.11A), and this induction of Myc was effectively abolished by PDK1 inhibitor BX912, but not by p110a inhibitor PIK90, which efficiently blocks PI3K downstream effector AKT phosphorylation (Figure 5.11B). To examine whether ectopic expression PDK1 induces endogenous Myc phosphorylation, two immortalized epithethial cell lines MCF-10A and HEK-TERV were stably introduced with PDK1. The immunoblotting analysis indicated that ectopic PDK1 can also induced endogenous Myc phosphorylation in these cells (Figure 5.11C). Collectively, these results demonstrate that PDK1 results in Myc phosphorylation and accumulation in epithelial cells.

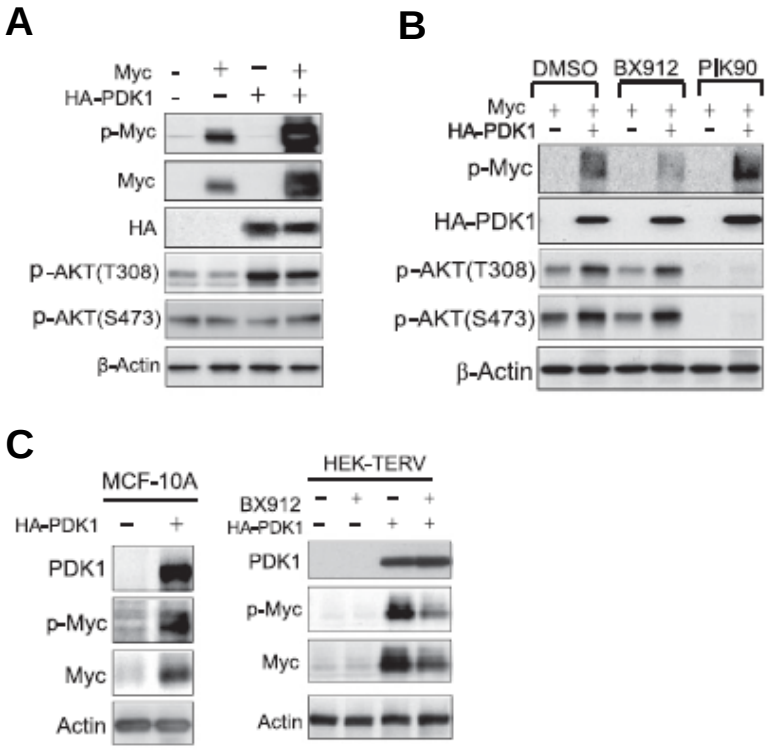


Figure 5.11 Etopic expression of PDK1 results in Myc phosphorylation

(A) Immunoblot analysis of Myc in 293T cells transfected with Myc, PDK1, or both.
(B) Immunoblot analysis of Myc in 293T cells transfected with Myc, PDK1, or both, treated with or without PDK1 inhibitor BX912, or PIK3CA inhibitor PIK90.
(C) Immunoblot analysis of endogenous Myc in MCF10A and HEK-TERV cells expressing exogenous PDK1.

5.2.4 *PPP2R2B* binds to and inhibits PDK1 activity

PPP2R2B binds to PDK1 protein in exogenous and endogenous system

PP2A regulates kinase phosphorylation through binding to and inhibiting the kinase activity (Sablina et al., 2007). We next tested the possibility that *PPP2R2B* may directly interact with PDK1 and modulate its activity. This hypothesis was confirmed by co-transfection of *PPP2R2B* and PDK1 plasmids in 293T cells followed by co-immunoprecipitation analysis with ectopic PDK1 and *PPP2R2B*-Myc (Figure5.12A). This interaction was further confirmed in DLD1-*PPP2R2B* cells in which Dox-induced *PPP2R2B* co-immunoprecipitated with endogenous PDK1 (Figure5.12B). To show the interaction between the endogenous PDK1 and *PPP2R2B* proteins, we took the advantage of HEK-TERV and HEK-TERV-*PPP2R2B* (B55β) shRNA cells and conducted the coimmunoprecipitation experiments using PDK1 and B55 antibody, respectively. The result shows that the endogenous PDK1 clearly interacted with total B55 and this interaction was largely diminished on B55β depletion (Figure5.12C). This result indicates that out of four B55 subunits B55β is the major one that interacts with PDK1.

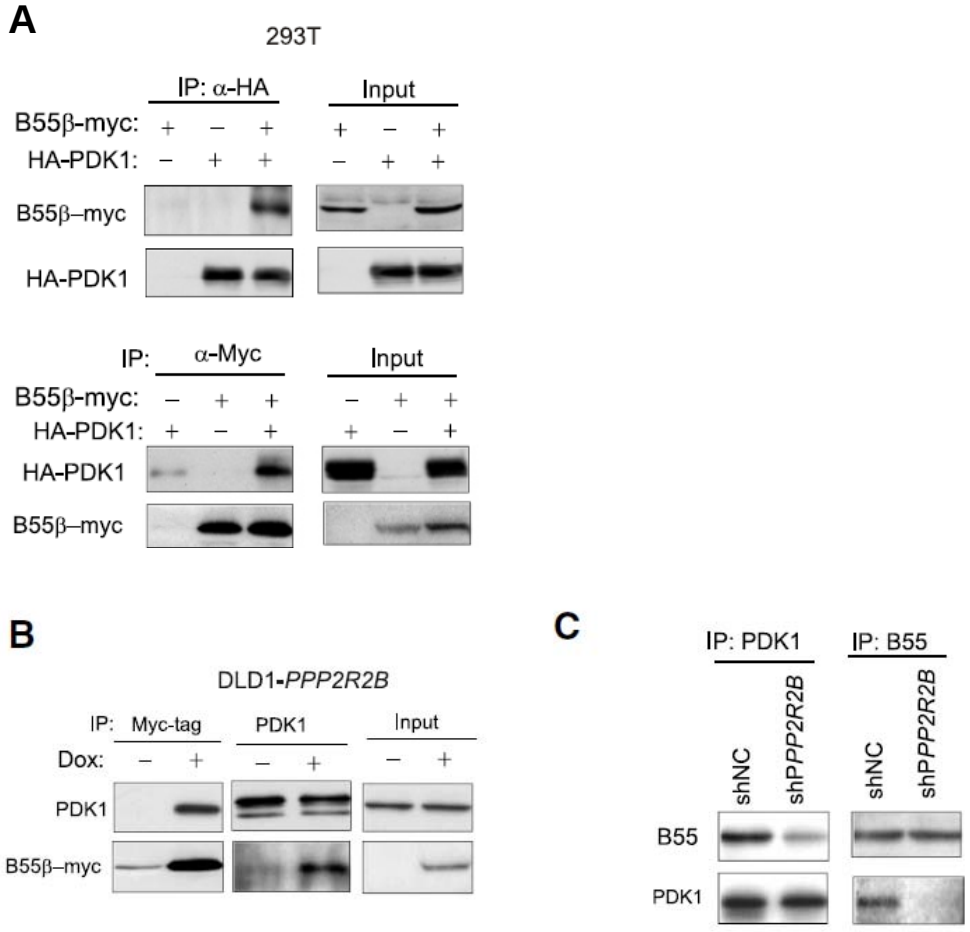


Figure 5.12 PPP2R2B interacts with PDK1

(A) Coimmunoprecipitation assay in 293T cells transfected with B55β-Myc, PDK1-HA, or both.
(B) Coimmunoprecipitation assays in DLD1-*PPP2R2B* cells. Cells were treated with or without Dox for 24 hr and B55b-Myc or PDK1 were pulled down and subjected to immunoblot analysis.
(C) Coimmunoprecipitation assays in HEKTERV and HEKTERV-sh*PPP2R2B* cells using B55 antibody and PDK1 antibody, respectively. The endogenous interaction between PDK1 and B55β was markedly reduced in HEKTERV expressing sh*PPP2R2B*.

PPP2R2B inhibits PDK1 membrane recruitment, resulting in inhibition of its activity

It is known that PDK1 needs to be recruited from the cytosol to the plasma membrane to activate its downstream targets (Kikani et al., 2005). Both *PPP2R2B* and PDK1 are predominantly located in the cytoplasm (Figure 5.13A and 13B). Thus, the cytoplasmic interaction of PDK1 with *PPP2R2B* may affect its membrane recruitment. Indeed, upon Dox treatment of DLD1-*PPP2R2B* cells, we detected the downregulation of both total and phosphorylated PDK1 in plasma membrane (Figure 5.14A). Moreover, in DLD1-*PPP2R2B* cells under serum starvation, ectopic PDK1-HA was mainly detected in the cytosol, but expressed in both cytosol and cell membrane upon serum stimulation, which was abolished when cells were treated with Dox to induce *PPP2R2B* (Figure 5.14B). As a consequence of this inhibitory effect on PDK1 membrane localization, we show that p-PKCζ(T410), a known PDK1 substrate was downregulated by *PPP2R2B* re-expression or PDK1 knockdown, although other examined PDK1 substrates did not seem to be significantly regulated by PDK1 in DLD1 cells and were thus not affected by *PPP2R2B* re-expression (Figure 5.14C). The overall result demonstrated that B55β-PP2A complex binds and inhibits PDK1 membrane recruitment, resulting in the inhibition of its activity toward its downstream substrates. Because Myc is accumulated mainly in the nucleus in response to rapamycin (Figure 5.14D), the effect of cytoplasmic B55β-PDK1 on Myc is most likely to be indirect and may route through PDK1 downstream kinase substrates. Taken together, these results demonstrate that *PPP2R2B*-PP2A complex binds to and inhibits PDK1 membrane

recruitment for activation.

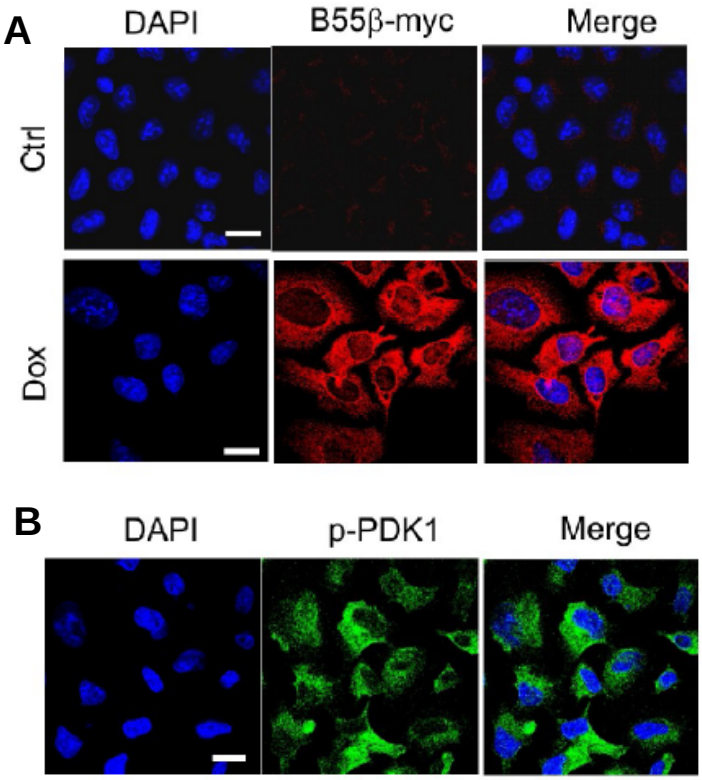


Figure 5.13 PPP2R2B, PDK1 and Myc cellular localization

Figure 5.13 *PPP2R2B*, PDK1 and Myc cellular localization

(A) DLD1-*PPP2R2B* cells were treated with Dox for 48h to induce *PPP2R2B* expression and cells were stained with anti-Myc (9E10) and DAPI to detect the localization of B55ß-Myc in DLD1 cells. Scale bars=10μm.
(B) DLD1 cells stained with anti-p-PDK1 (S241) to detect the localization. Scale bars=10μm.

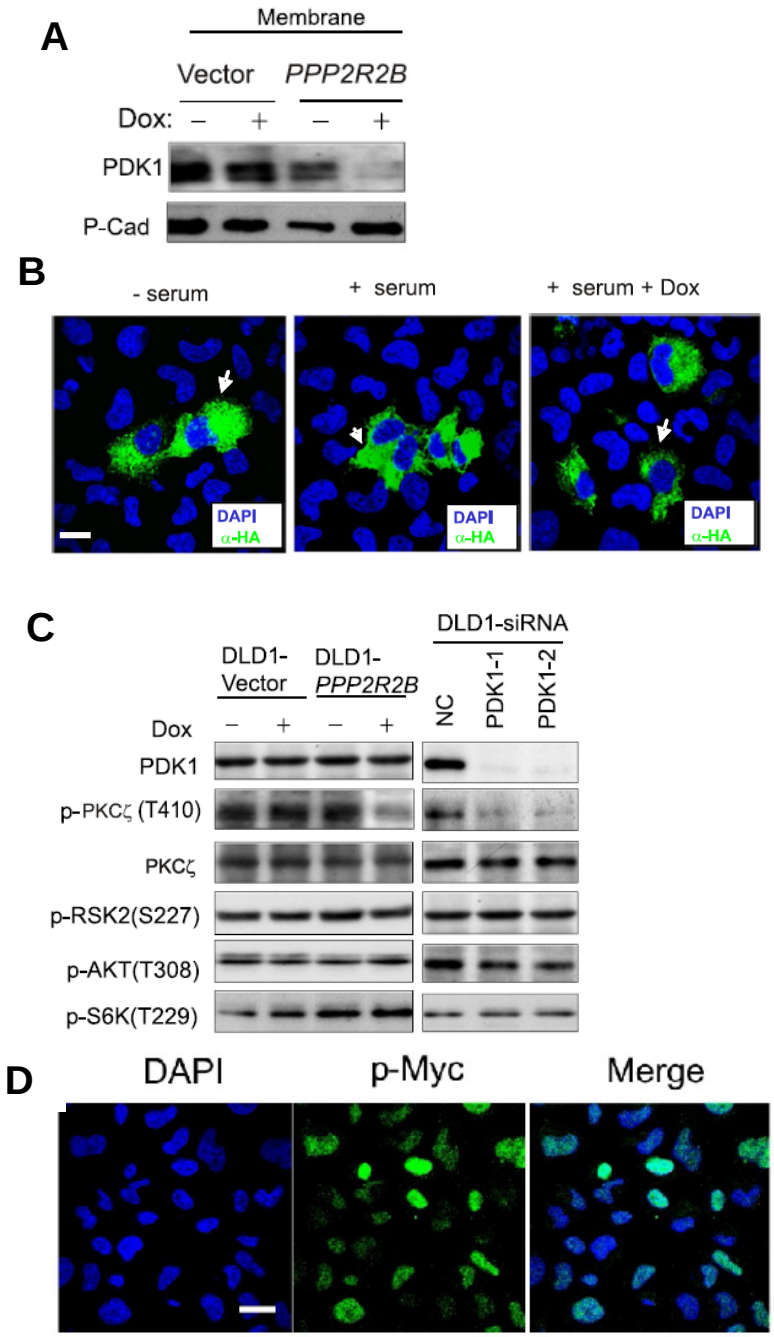


Figure 5.14 PPP2R2B inhibits PDK1 Membrane Localization

(A) Immunoblot analysis of the membrane fractions on PDK1 prepared from DLD-*PPP2R2B* cells treated with or without Dox for 48 hr.
(B) Immunofluorescence for PDK1-HA in DLD1-*PPP2R2B* with or without serum and Dox treatment. Scale bar, 10μm.
(C) Immunoblot analysis of known PDK1 substrates on *PPP2R2B* re-expression or PDK1 knockdown in DLD1 cells.
(D) DLD1 cells treated with rapamycin for 48 h and stained with anti-p-Myc(T58/S62). Scale bars=10μm.

5.2.5 Inhibition of PDK1 and Myc, but not PIK3CA and AKT, sensitizes therapeutic response of rapamycin

Knockdown of PDK1 and Myc sensitizes rapamycin response in CRC

Rapamycin induces Myc phosphorylation through PDK1 pathway but not PIK3CA-AKT pathway. To evaluate the underlying mechanisms how the two distinct signaling pathways contribute to rapamycin resistance, We knockdown the components of PDK1 and PIK3CA pathways using siRNA in HCT116 cells and checked the rapamycin sensitivity. Cells were treated with rapamycin for 5 days and measured for cell viability. As shown in Figure 5.15A, PDK1 or Myc knockdown resulted in markedly increased sensitivity of rapamycin in HCT116 cells, while *PIK3CA* or AKT knockdown did not give rise to a similar effect. To confirm the findings in HCT116 cells, a retroviral shRNA targeting PDK1 was introduced into SW480 cells and selected for stable cell lines. The stable knockdown cells were treated with rapamycin and cell viability was measured. As shown in Figure 5.15B, the similar effect of PDK1 ablation on Myc and rapamycin sensitivity was further validated in SW480 cells expressing a retroviral PDK1 shRNA. These results support a causal relationship between PDK1-Myc induction and rapamycin resistance in CRC cells. Moreover, the data argue for a more important role of PDK1-Myc signaling, as compared to *PIK3CA*-AKT signaling, in rapamycin resistance in CRC cells.

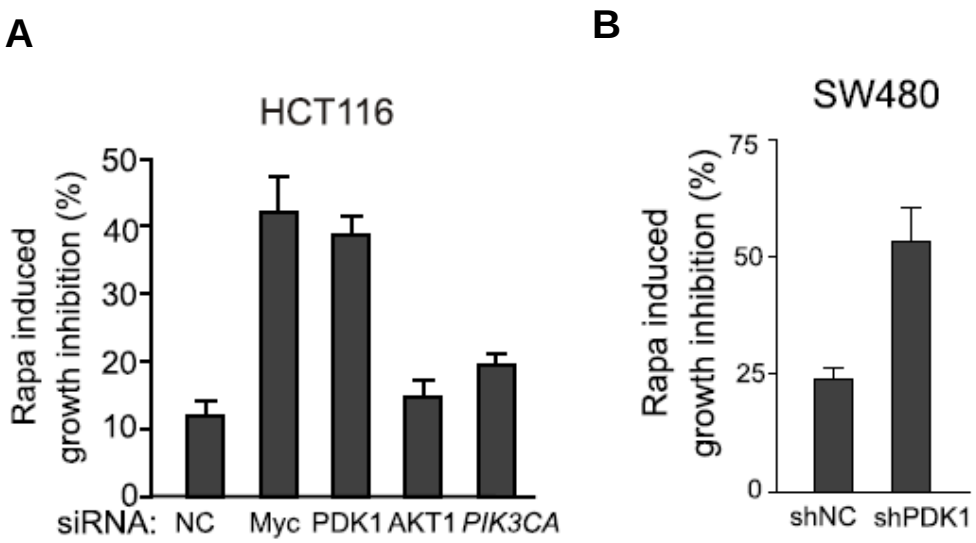


Figure 5.15 PDK1 and Myc knockdown sensitizes rapamycin response in CRC

(A) HCT116 cells were transfected with siRNAs targeting Myc, PDK1, AKT1, or PIK3CA for 48 hr, and then treated with 100 nM rapamycin for 5 days. The graph bars show the rapamycin-induced growth inhibition relative to nontreated cells.

(B) Rapamycin-induced growth inhibition in SW480 cells expressing PDK1 shRNA or a negative control shRNA.

Pharmacological inhibition of PDK1 and rapamycin synergistically induced cell cycle arrest in G2/M phase

Identification of *PPP2R2B*-regulated PDK1 suggests a practical approach for overcoming rapamycin resistance, which may be achieved through pharmacological inhibition of PDK1. To examine whether the effect of PDK1 inhibition would be the same as *PPP2R2B* re-expression in colon cancer cells, we compared the changes of morphology in SW480 cells treated with PDK1 siRNA and PDK1 inhibitor BX912. The microscope images indicated that PDK1 shRNA or BX912, but not PIK90, induced a morphological change similar to that seen upon *PPP2R2B* re-expression in SW480 cells. Moreover, BX912, but not PIK90, synergized with rapamycin to induce strong G2/M arrest in both DLD1 and SW480 cells (Figure 5.16B), which is again remarkably consistent with the synergistic effect of *PPP2R2B* and rapamycin on G2/M cell cycle induction. Thus, pharmacologic inhibition of PDK1, but not *PIK3CA*, has phenocopied the effect of *PPP2R2B* re-expression, further supporting the genetic interaction between *PPP2R2B* and PDK1.

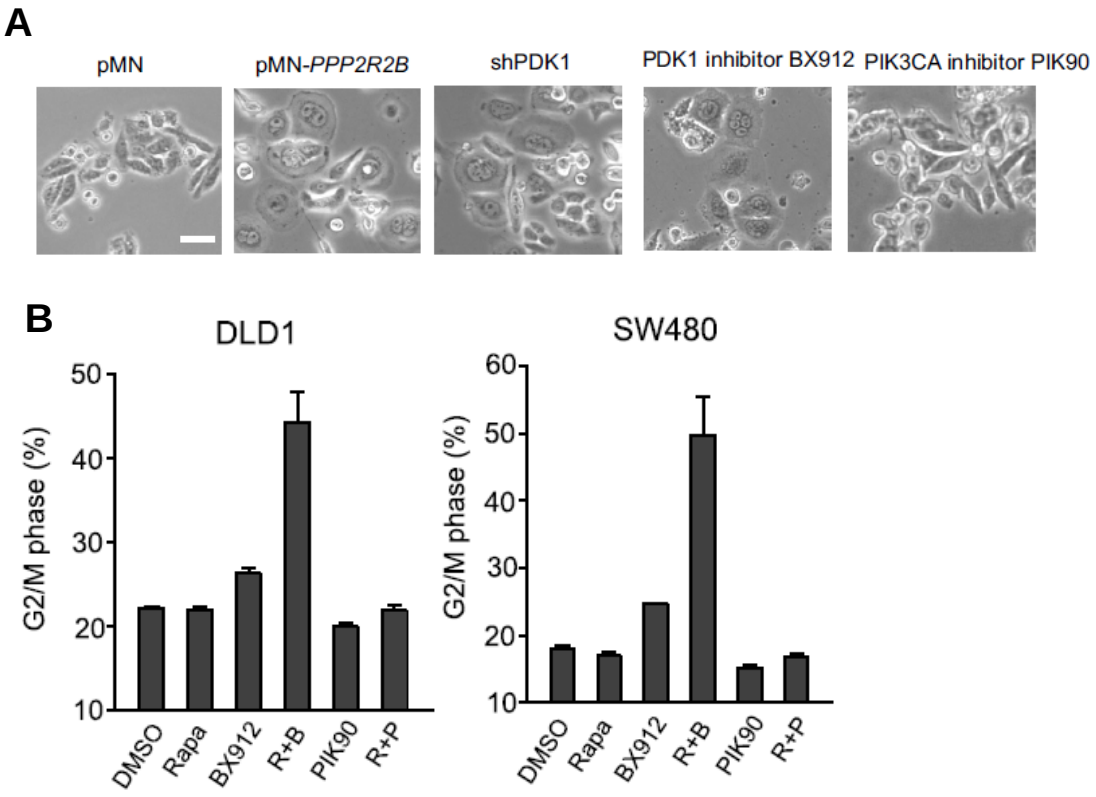


Figure 5.16 PDK1 inhibition results in similar effects of PPP2R2B re-expression in CRC

(A) Morphologies of SW480 cells under indicated treatments. Scale bars represent 10μm.

(B) G2/M phase arrest in SW480 and DLD1 cells induced by rapamycin (10 nM), BX912 (2.5 μM), PIK90 (5 μM), single or in combinations, assessed by PI staining and FACS analysis. Data are presented as mean ± SD of the percentages of cells arrested in G2/M.

Pharmacological inhibition of PDK1 can overcome rapamycin resistance

To further investigate the synergistic effects of inhibition of PDK1 with rapamycin, we carried out the cell viability and colony formation assay in both DLD1 and SW480 cells treated with PDK1 inhibitor in the presence or absence of rapamycin. We observed a synergistic loss of cell viability with combined treatment of BX912 and rapamycin in cells for a 5-day cell viability assay (Figure5.17C) and a 2-week colony formation assay (Figure5.17D). These findings support a model in which the loss of *PPP2R2B* in CRC results in activation of PDK1-dependent Myc phosphorylation in response to rapamycin treatment, leading to rapamycin resistance in a *PIK3CA*-AKT independent manner (Figure5.18). Pharmacologic inhibition of PDK1 can overcome rapamycin resistance by preventing the Myc phosphorylation, pointing out a potential combination strategy for CRC treatment.

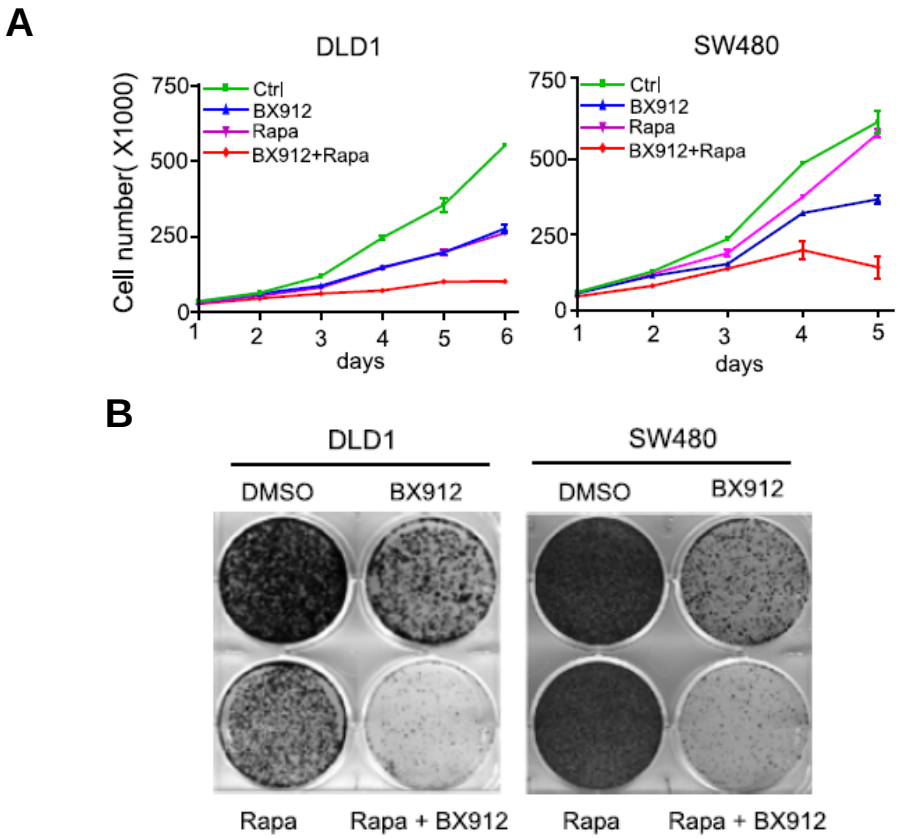


Figure 5.17 Pharmacologic Inhibition of PDK1-Myc Signaling Overcomes Rapamycin Resistance

(A) Cell viability of DLD1 and SW480 cells treated with BX912 (2.5μM), rapamycin (10nM), or both for indicated days.
(B) Dense foci formation for 14 days on a monolayer of DLD1 and SW480 cells treated as (A).

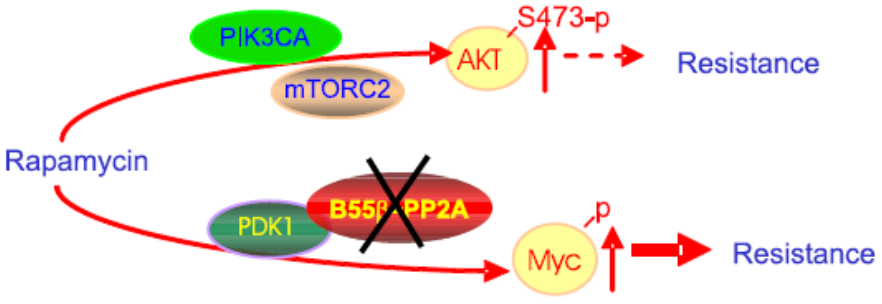


Figure 5.18 A model indicating a role of B55β-regulated PDK1-Myc pathway in modulating rapamycin response.

Loss of *PPP2R2B* expression in CRC results in induction of PDK1-dependent Myc phosphorylation by rapamycin, conferring rapamycin resistance.

5.3 Discussion

In this study, we have revealed a novel function of PDK1 toward Myc regulation in response to rapamycin, which is correlated with *PPP2R2B* expression. This study has showed that *PPP2R2B*-associated PP2A complex controls PDK1-Myc signaling and modulates rapamycin sensitivity in colorectal cancer. This PDK1-Myc signaling is independent of *PIK3CA-AKT* and may constitute an alternative feedback mechanism leading to rapamycin resistance. Although *PIK3CA* and mTORC2-dependent AKT S473 phosphorylation has been suggested to be one of crucial feedback mechanisms accounting for the rapamycin resistance in certain contexts (O'Reilly et al., 2006; Sarbassov et al., 2006), this pathway in CRC may not be as critical as rapamycin resistance that is induced through Myc phosphorylation because knockdown of *PIK3CA* or AKT, does not sensitize cells to rapamycin treatment. As such, we propose an AKT-independent signaling pathway comprised of PDK1 and Myc phosphorylation contributing to rapamycin resistance in CRC, due to loss of *PPP2R2B*. This notion seems to be consistent with a recent finding that AKT is often not required for proliferation of cancer cells with activated PI3K pathway (Vasudevan et al., 2009). Thus, the identification of a PDK1-Myc signaling regulated by *PPP2R2B* widens our understanding of cancer cell growth control and rapamycin resistance and emphasizes the importance of epigenetic mechanisms in regulating oncogenic signaling and therapeutic response. However, although we demonstrate that Myc as a downstream target of PDK1, PDK1 may not regulate Myc directly. It is possible that PDK1 may route through other downstream kinases to affect Myc

phosphorylation. The precise feedback mechanism leading to the induction of Myc phosphorylation by rapamycin remains elusive and warrants further investigation.

The dual PI3K-mTOR inhibitors such as PI-103 and NVP-BEZ235 also induce PDK1-dependent Myc phosphorylation and Myc protein accumulation in colon cancer cells, though these dual inhibitors are able to overcome the activation of AKT phosphorylation (Figure 5.9B). These findings point to significant concerns as these inhibitors are being tested in clinical trials in various human tumors (Fan et al., 2006; Serra et al., 2008). One would expect that this compensatory activation of Myc signaling, if validated in the primary tumors, may eventually lead to acquired resistance and patient relapse. In agreement with our findings, a recent report also suggest that aberrant evelation of Myc by gene amplification represents a potential resistance mechanism with respect to current PI3K-targeted therapies in clinical trials, which is mediated through a PI3K-independent mechanism (Ilic et al., 2011; Liu et al., 2011). Most importantly, considering a potential role of Myc in cancer stem cells (Cairo et al., 2008; Kim et al., 2010; Wong et al., 2008), our study suggests that caution has to be taken during the development of clinical trials of PI3K-mTOR inhibitors in human cancers such as colon, bladder, brain, and esophagus tumors in which *PPP2R2B* might be downregulated. In this aspect, expression levels of *PPP2R2B* or its promoter DNA methylation status might be a useful predictive marker to guide the clinical trials of mTOR inhibitors, and Myc phosphorylation as a surrogate marker to asses the potential adverse effect of mTOR inhibitors in these tumors. To overcome this potential problem, a combinational

approach involving PDK1 inhibition might be effective in overcoming Myc activation and resistance to mTOR inhibitors, and to produce a more durable cancer regression. Of notice, the PDK1-mediated regulation of Myc might not be direct and other kinases downstream of PDK1 might be involved in this process. Thus, Identification of additional kinases in this pathway will provide additional therapeutic targets and inform new therapeutic opportunities.

Our data support PDK1 as a therapeutic target in CRC, as removal of PDK1 reduces Myc signaling and alleviate rapamycin resistance. This notion was further illustrated using a small molecule PDK1 inhibitor BX912, which is able to abolish rapamycin–induced Myc phosphorylation and thus synergizes with rapamycin in CRC. Notably, the PDK1 inhibitor as an anti-cancer agent has been shown to be effective *in vitro* and *in vivo* in cancer (Maurer et al., 2009; Peifer and Alessi, 2008) and is currently under clinical development. Therefore, we propose that its combination with rapamycin-based mTORC1 inhibitors may become a useful option for targeted therapy of CRC.

Although the rapamycin resistance is well-known to be caused in part by feedback loops that activate PI3K-AKT and ERK-MAPK signaling, our studies suggest that PDK1-dependent Myc phosphorylation may provide an alternative mechanism conferring rapamycin resistance. In fact, rapamycin could not induce MAPK phosphorylation in colon cancer cells and rapamycin-induced Myc phosphorylation seems play a more effective role than AKT phosphorylation in rapamycin resistance, as knockdown of Myc sensitized rapamycin response, which

was not observed upon AKT knockdown (Figure 5.16A). As such, we defined a novel oncogenic signaling module comprised of PDK1 and myc phosphorylation that contributes to rapamycin resistance in colorectal tumors in which *PPP2R2B* is lost.

In summary, we have demonstrated that Myc induction is independent of AKT phosphorylation and thus provides a parallel pathway to mediate rapamycin resistance. Rapamycin-induced Myc phosphorylation does not require PIK3CA and PIK3CB, which are required for the AKT S-473 phosphorylation by rapamycin. We also noticed that in both rapamycin-sensitive and -resistant cell lines AKT is consistently upregulated, while Myc induction is only observed in *PPP2R2B* silencing cells that are resistant to rapamycin, but not in rapamycin sensitive and *PPP2R2B* expressing cells. This highlights a distinct role of Myc phosphorylation and protein accumulation by PDK1 in mediating rapamycin sensitivity. Thus, this alternative feedback mechanism present as promising therapeutic targets for overcoming rapamycin resistance in certain human cancer such as colon cancer.

6 Chapter VI: Discussion

In this thesis, by using integrative genomic and epigenomic analysis we identified and characterized 203 CMS genes with clinical relevance and potential tumor suppressor functions in colorectal cancer. Among these CMS genes, *PPP2R2B*, one of the regulatory B subunit of PP2A, was found to be epigenetically inactivated by DNA methylation in CRC. Loss of *PPP2R2B* causes inactivation of PP2A complex and promotes PDK1-Myc signaling, which is required for CRC cell survival and proliferation. In agreement with this, gain-of-function analysis of *PPP2R2B* revealed its inhibition of PDK1-Myc axis as a potential tumor suppressor mechanism. Moreover, remarkable loss of *PPP2R2B*-associated PP2A function in CRC conferred a strong activation of oncogenic Myc phosphorylation in response to mTORC1 inhibitor rapamycin, leading to rapamycin resistance. Taken together, these findings suggest that aberrant activation of PDK1-Myc is a potential mechanism by which tumor cells develop resistance to mTOR inhibition, and thus combination therapies targeting both mTOR and PDK1 may be necessary to circumvent resistance to PI3K/mTOR-targeted therapy.

6.1 Meta-analysis of genomic and epigenomic data reveals CMS gene set in colon cancer

Aberrant epigenetic silencing is a prevalent mechanism that could lead to inactivation of tumor suppressors in human malignancy (Chan et al., 2008; Jiang et al., 2008). One of the predominant modes of epigenetic alterations in cancer is gene silencing via DNA methylation of CpG islands in promoter region(Esteller et al.,

2001; Jones and Baylin, 2007). Increasing evidences have shown that DNA hypermethylation results in abnormal silencing of a number of tumor suppressors in many solid tumors (Esteller, 2008). Genome-wide identification of DNA methylation silencing genes and subsequent functional characterization of the tumor suppressor genes in several types of cancers have been performed in previous reports (Schuebel et al., 2007; Suzuki et al., 2002; Suzuki et al., 2004). However, the full spectrum of tumor suppressor genes silenced by DNA methylation in CRC is not well documented.

In chapter III, by adopting combination analysis of transcriptomic profiling and ChIP-seq mapping, we have identified clinically relevant tumor suppressor genes that silenced by DNA hypermethylation in CRC. Many of the CMS genes were revealed to have functions relevant to tumor biology, cell proliferation, and metastasis. Functional studies indicate that some of these genes work as tumor suppressors, leading to growth inhibition in cancer cells. Discovery of tumor suppressor genes and their related oncogenic signaling pathways that are clinically relevant and functionally important is critical for the development of novel therapeutics. This study provided a comprehensive approach to identify and characterize the silenced tumor suppressor genes in CRC, which has enhanced our understanding of the process of tumorigenesis. Previous studies have shown that tumor suppressor genes are frequently silenced epigenetically in colon cancer cell lines and primary tumors (Suzuki et al., 2002). In agreement with this, a large number of CMS genes were uncovered in this study. In addition, many of these

genes were previously reported as putative tumor suppressor genes in colorectal cancer, such as *SOX17* and *SFRP1*. Thus, this study has significantly uncovered a wide array of novel silenced genes that potentially act as tumor suppressors in colon cancer.

6.2 *PPP2R2B*-associated PP2A complex functions as a tumor suppressor

Aberrant DNA methylation-mediated silencing of tumor suppressor genes often resulted in the dysregulation of oncogenic pathways and thus plays as an important mechanism in tumorigenesis and resistance to anticancer therapy (Baylin, 2011). Increasing evidences have validated that epigenetic alterations collaborate with genetic changes to disrupt the core pathways in cancer (2011; Wood et al., 2007; Yi et al., 2011). However, most of these studies only focus on the identification of molecular abnormalities that influence clinical outcome, the relationship between epigenetic alteration and therapeutic resistance is under studied.

In chapter IV, a specific PP2A regulatory B subunit which encoded B55β was identified to be epigenetically inactivated in both CRC cell lines and patient-derived CRC samples. Gain- and loss-of-function analyses reveal that *PPP2R2B* forms an active PP2A complex, which works as a tumor suppressor in CRC. Therefore, one of the important contributions of this study is the discovery of a potential mechanism conferring the inactivation of PP2A tumor suppressors in colon and other human cancers. The low frequency of genetic alterations in PP2A subunits is consistent with

the previous studies, which indicated that tumor suppressors are mainly inactivated by epigenetic changes, such as DNA methylation, rather than genetic mutations (Baylin and Ohm, 2006; Schuebel et al., 2007). Our study indicates that the loss of *PPP2R2B* occurs in >90% of colorectal tumor samples was mediated by epigenetic events, implying that DNA hypermethylation might be more prevalent mechanism leading to loss of PP2A tumor suppressor function in colon cancer.

Re-expression of *PPP2R2B* could reverse the cancer phenotypes through restoration of its associated-PP2A complex tumor suppressor activity. However, the underlying mechanism in which PP2A works as tumor suppressor in CRC is largely unknown. Given the substrate diversity and specificity of PP2A regulatory subunits, we screened a panel of key components of core oncogenic pathways in CRC and revealed that the loss of *PPP2R2B* associated specific PP2A complex mainly results in deregulation of Myc and p70S6K. Genetic disruption of Myc and p70S6K indicated that Myc appears to be a crucial downstream target of *PPP2R2B*-associated PP2A and inactivation of Myc by PP2A-*PPP2R2B* is consistent with the strong growth inhibition effect of *PPP2R2B* on CRC cells. Although a distinct PP2A subunit B56α has been shown to associate with Myc and regulates its stability (Arnold and Sears, 2006; Junttila et al., 2007; Yeh et al., 2004), we show that *PPP2R2B*-associated PP2A complex indirectly regulate Myc phosphorylation and protein accumulation. Thus, the loss of *PPP2R2B* provides an additional mechanism leading to deregulation of Myc in CRC.

6.3 Rapamycin-induced Myc phosphorylation as a rapamycin resistance mechanism

The PI3K/AKT signaling impacts on cancer cell growth, survival, and metabolism. mTOR is the key downstream component of this pathway, which is hyperactivated in many types of cancer (Sabatini, 2006). Misregulated mTOR activity is a common feature of most cancers (Alessi et al., 2009); therefore, the involvement of mTOR in tumorigenesis has been extensively investigated during the past years. Several mTOR inhibitors, such as rapamycin and its analogs, have been studied in clinical trial, including renal cell carcinoma, lymphoma, and breast cancer (Chan et al., 2005; Hudes et al., 2007; Witzig et al., 2005). However, the clinical trials evaluating the mTORC1 inhibitor rapamycin as anticancer agent have met with limited success and like most of the targeted therapies are inevitably followed by acquired drug resistance (Guertin and Sabatini, 2009). This limited success can be explained by the negative feedback loops that resulted in the hyperactivation of PI3K/AKT or ERK-MAPK signaling pathways, while additional mechanisms contributing to the resistance to mTOR inhibitors may also exist and remain to be identified. Considering this, it is imperative to identify the drug resistance mechanism and discover the relevant biomarkers that can be used to predict the response of human cancers to mTOR inhibitors in order to optimize their utility and improve clinical outcomes.

In chapter V, we found that rapamycin treatment not only induced AKT activation, but also led to a strong Myc phosphorylation in colorectal cancers cells.

This rapamycin-induced Myc phosphorylation in colon cancer cells is not routed through PI3K, but is dependent on PDK1, a master kinase often linked to AKT activation. Intriguingly, PDK1 inhibition by either gene knockdown or small molecule kinase inhibitor does not affect AKT phosphorylation in colon cancer cells, but can markedly abolish Myc phosphorylation, leading to enhanced sensitivity to rapamycin in CRC. This suggests that mTORC1 inhibition by rapamycin may trigger a separate compensatory mechanism involving PDK1-Myc signaling to attenuate rapamycin response. Interestingly, several recent studies have reported similar mechanisms of resistance to PI3K-targeted therapy. In one study, tumor induced by Myc activation was shown to be highly resistant to BEZ235, a dual PI3K and mTORC1/2 inhibitor, compared to PTEN-deficient-induced tumor (Carver et al., 2011). Likewise, Myc elevation in PI3K-driven mammary tumors represents a potential mechanism of resistance to PI3K inhibition (Ilic et al., 2011; Liu et al., 2011). These studies, along with our present study, strongly suggest that aberrant activation of Myc may contribute to acquired resistance in PI3K/mTOR-targeted therapy. Although PI3K-dependent AKT phosphorylation has been suggested to be a crucial mechanism accounting for the rapamycin resistance in other contexts (O'Reilly et al., 2006; Sarbassov et al., 2006), this pathway does not seem to be crucial in colon cancer as we found that knockdown of PIK3CA and AKT did not significantly sensitize rapamycin-induced growth inhibition. Furthermore, this study indicated that the rapamycin-induced Myc phosphorylation in colon cancer appears to be a result of the epigenetically loss of expression of *PPP2R2B* and inactivation of

its associated PP2A complex. In colon cancer cells with restored expression of PP2A-*PPP2R2B* or in human cancer cells that do not acquire silencing of PP2A-*PPP2R2B*, rapamycin does not seem to induce Myc phosphorylation and these cells were found to be more sensitive to rapamycin. Moreover, this study also demonstrated that *PPP2R2B*-assocated PP2A complex is physically interacting with PDK1 and functionally mitigates PDK1-directed Myc phosphorylation. Given the high frequency of *PPP2R2B* silencing (>90%) in CRC, we proposed a model for rapamycin resistance in CRC. As shown in Figure 6, rapamaycin induces PDK1-dependent Myc phosphorylation, which is negatively regulated by *PPP2R2B*-PP2A complex; while epigenetic inactivation of *PPP2R2B* results in activation of PDK1-Myc signaling upon rapamycin treatment, leading to rapamycin resistance. Taken together, these findings identified a crucial role of PP2A-*PPP2R2B* in determining the activity of PDK1-directed Myc signaling response to rapamycin and thus the cellular sensitivity to mTOR inhibitors.

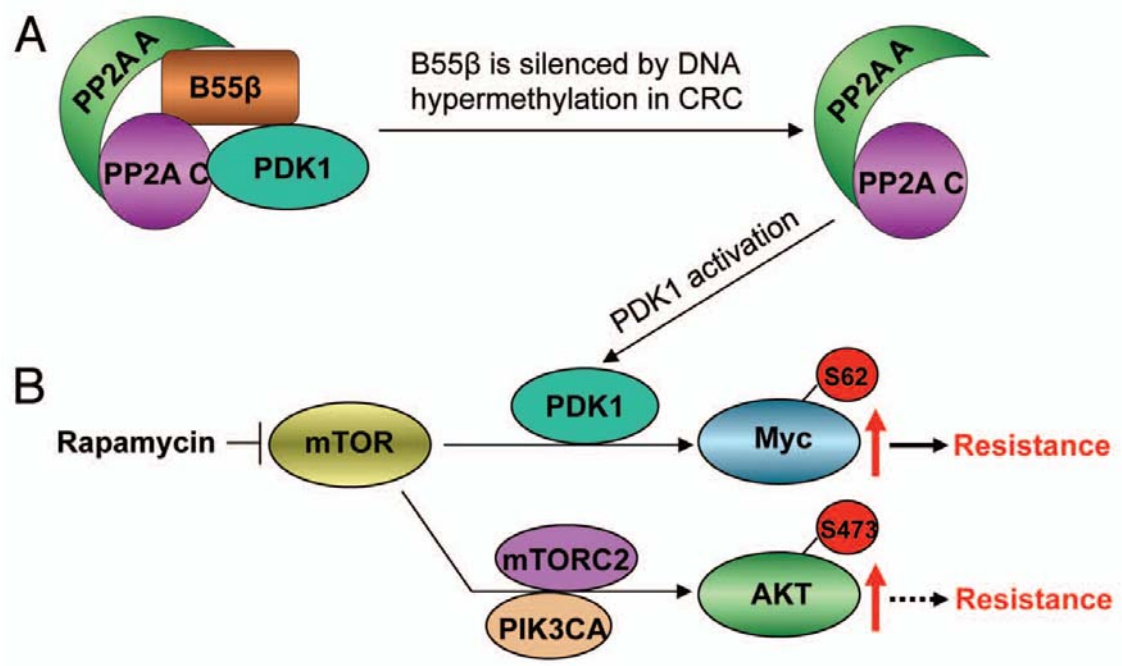


Figure 6.1 The role of PP2A-B55β-regulated PDK1-Myc pathway in modulating rapamycin response.

(A) The epigenetic inactivation of *PPP2R2B* (encoding B55β) by promoter DNA hypermethylation in colorectal cancer (CRC) disrupts the PP2A-B55β function towards inhibition of PDK1-Myc signaling. (B) Rapamaycin induces PDK1-dependent Myc phosphorylation in CRC due to loss of PP2A-B55β, which is parallel to PIK3CA/mTORC2-dependent AKT S473 phosphorylation, attenuating therapeutic effect of rapamycin.

6.4 Potenital clinical aplications of this study

This thesis has revealed several implications for clinical application of rapamycin derivatives in human cancer therapy. Firstly, *PPP2R2B* may be used as a predictive biomarker for clinical outcome of mTOR inhibitors in cancer therapy. Several rapamycin analogs are currently under the clinical development. Unfortunately, clinical updates indicate that rapamycin shows promising results only towards a few types of cancers, particularly B-cell lymphoma, endometrial cancer, and renal cell carcinoma (Easton and Houghton, 2006; Faivre et al., 2006; Granville et al., 2006). Overall, the therapeutic response to rapamycin is highly variable, indicating the strong need for biomarkers that are capable of accurately predicting the therapeutic effect of rapamycin. As we discussed earlier, the silencing of *PPP2R2B* by DNA methylation occurs in more than 90% of colon primary tumor and this epigenetic event impacts the sensitivity of cancer cells to mTOR inhibitors. In this study, a strong correlation of the expression of *PPP2R2B* with the response of rapamycin has been revealed. Thus, this finding may have important implications for clinical trials of rapamycin derivatives in human cancer. As such *PPP2R2B* could serve as one of the predictive markers for prognosis of CRC patients as well as for patient selection in the clinical trials.

Secondly, Myc phosphorylation can be used as a surrogate marker to evaluate the drug response of rapamycin. In this study, we showed that rapamycin treatment resulted in the induction of AKT S473 phosphorylation through a well-known feedback mechanism in both sensitive and resistant cancer cells. In contrast, the

rapamycin-induced Myc phosphorylation only occurs in resistant cell lines which lack of *PPP2R2B* expression compared to the sensitive cancer cells. Thus, these findings suggest that Myc phosphorylation induced by rapamycin may serves as prognostic biomarker for mTOR inhibitors in the clinical outcome.

Finally, this study has highlighted that PDK1 could become a therapeutic target in cancer therapy. An additional therapeutic application of this study is the identification of a new strategy to overcome resistance to mTOR-targeted therapy in CRC. Our data show that ectopic expression of PDK1 promotes Myc phoshorylation and protein accumulation; conversely, pharmacological or genetic removal of PDK1 reduces Myc signaling and alleviates rapamycin resistance. This notion was further illustrated using a small molecule PDK1 inhibitor BX912, which is able to abolish rapamycin–induced Myc phosphorylation and thus synergizes with rapamycin in CRC. In conjunction with these findings, it was reported that genetic ablation of PDK1 in human colorectal cancer resulted in markedly reduced proliferation and metastasis (Ericson et al., 2010). In addition, the PDK1 inhibitor has been shown to be an effective anti-cancer agent *in vitro* and *in vivo* (Maurer et al., 2009; Peifer and Alessi, 2008) and is currently under clinical development. To overcome the resistance of mTOR inhibitors in targeted therapy, a combination approach involving PDK1 inhibition might be appropriate to overcome Myc activation and resistance to mTOR inhibitors, and to produce a more durable cancer regression. Therefore, we propose that its combination with rapamycin-based mTORC1 inhibitors could be a better option for targeted therapy of CRC. Taken together, this study provides an illustration

of epigenetic control of important oncogenic signaling pathways and identifies a molecular determinant and a potential biomarker of rapamycin sensitivity.

6.5 Future directions

This study provides a genome-wide mapping approach to discover epigenetically silencing genes by DNA methylation. The subsequent functional studies have helped us to identify a panel of putative tumor suppressor genes in CRC. However, the underlying mechanism(s) of how these CMS genes work as tumor suppressors remains elusive. Although identification and characterization of these CMS genes could provide new insights into cancer development, detailed molecular mechanisms of these CMS genes need to be further investigated. For example, although the differentiation transcription factor *HAND1* was identified as a tumor suppressor gene in this study and other reports (Martinez Hoyos et al., 2009; Tellez et al., 2009), the mechanism is still unknown. Gain-of-function analysis of *HAND1* in inducible cell systems would provide detailed information of the tumor suppressor role of *HAND1* in CRC. For instance, the gene expression data could be useful for identification of *HAND1* transcriptional targets and its associated regulatory network. On top of that, some of CMS genes encode protein phosphatases, which regulate a variety of kinases involved in oncogenic pathways. Epigenetically loss of these protein phosphatases may play an important role in the deregulation of oncogenic phosphorylation cascades and result in tumorigenesis. Therefore, functional investigation of these phosphatases and identification of the substrates or kinases of

these phosphatases, would provide new therapeutic targets in cancer treatment.

In this study, we have revealed aberrant activation of PDK1-Myc signaling in colorectal cancer (CRC) and its association with the loss of PP2A-B55β phosphatase complex. Although co-immunoprocipitation results show that PDK1 indirectly promotes Myc accumulation, the precise mechanism how PDK1 regulates Myc is unclear. To understand the mechanisms, PDK1-transformed cell system would be set up and used as a model system for screening of the downstream mediators of PDK1 for Myc regulation. By using this model system, new compounds that could more specifically target PDK1 would be developed.

Clinically, this study reveals a potential resistance mechanism with respect to current mTOR-targeted therapies in clinical trials. The positive regulation of PDK1 toward Myc signaling pathway determines the response of rapamycin in cancer cells. Thus, it would be worth to examine the value of *PPP2R2B* as a predictive biomarker for rapamycin treatment in the clinical trial studies. Therefore, preclinical trials based on xenograft animal studies should be carried out to check the anti-tumor effects of combined treatment of PDK1 inhibitor and mTOR inhibitor.

Reference:

(2011). Integrated genomic analyses of ovarian carcinoma. Nature *474*, 609-615.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J *15*, 6541-6551.

Alessi, D.R., Pearce, L.R., and Garcia-Martinez, J.M. (2009). New insights into mTOR signaling: mTORC2 and beyond. Sci Signal *2*, pe27.

Andrabi, S., Gjoerup, O.V., Kean, J.A., Roberts, T.M., and Schaffhausen, B. (2007). Protein phosphatase 2A regulates life and death decisions via Akt in a context-dependent manner. Proc Natl Acad Sci U S A *104*, 19011-19016.

Arnold, H.K., and Sears, R.C. (2006). Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. Mol Cell Biol *26*, 2832-2844.

Arnold, H.K., and Sears, R.C. (2008). A tumor suppressor role for PP2A-B56alpha through negative regulation of c-Myc and other key oncoproteins. Cancer Metastasis Rev *27*, 147-158.

Arnold, H.K., Zhang, X., Daniel, C.J., Tibbitts, D., Escamilla-Powers, J., Farrell, A., Tokarz, S., Morgan, C., and Sears, R.C. (2009). The Axin1 scaffold protein promotes formation of a degradation complex for c-Myc. Embo J *28*, 500-512.

Arroyo, J.D., and Hahn, W.C. (2005). Involvement of PP2A in viral and cellular transformation. Oncogene *24*, 7746-7755.

Baylin, S.B. (2011). Resistance, epigenetics and the cancer ecosystem. Nat Med *17*, 288-289.

Baylin, S.B., Belinsky, S.A., and Herman, J.G. (2000). Aberrant methylation of gene promoters in cancer---concepts, misconcepts, and promise. J Natl Cancer Inst *92*, 1460-1461.

Baylin, S.B., and Ohm, J.E. (2006). Epigenetic gene silencing in cancer - a

mechanism for early oncogenic pathway addiction? Nat Rev Cancer *6*, 107-116.

Berger, A.H., Knudson, A.G., and Pandolfi, P.P. (2011). A continuum model for tumour suppression. Nature *476*, 163-169.

Berns, K., Horlings, H.M., Hennessy, B.T., Madiredjo, M., Hijmans, E.M., Beelen, K., Linn, S.C., Gonzalez-Angulo, A.M., Stemke-Hale, K., Hauptmann, M.*, et al.* (2007). A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell *12*, 395-402.

Beroukhim, R., Mermel, C.H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Barretina, J., Boehm, J.S., Dobson, J., Urashima, M.*, et al.* (2010). The landscape of somatic copy-number alteration across human cancers. Nature *463*, 899-905.

Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S., O'Reilly, T., Natt, F., Hall, J., Lane, H.A., and Thomas, G. (2005). The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell *120*, 747-759.

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355-365.

Brachmann, S.M., Hofmann, I., Schnell, C., Fritsch, C., Wee, S., Lane, H., Wang, S., Garcia-Echeverria, C., and Maira, S.M. (2009). Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. Proc Natl Acad Sci U S A *106*, 22299-22304.

Cairo, S., Armengol, C., De Reynies, A., Wei, Y., Thomas, E., Renard, C.A., Goga, A., Balakrishnan, A., Semeraro, M., Gresh, L.*, et al.* (2008). Hepatic stem-like phenotype and interplay of Wnt/beta-catenin and Myc signaling in aggressive childhood liver cancer. Cancer Cell *14*, 471-484.

Calin, G.A., di Iasio, M.G., Caprini, E., Vorechovsky, I., Natali, P.G., Sozzi, G., Croce, C.M., Barbanti-Brodano, G., Russo, G., and Negrini, M. (2000). Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms. Oncogene *19*, 1191-1195.

Cappellen, D., Schlange, T., Bauer, M., Maurer, F., and Hynes, N.E. (2007). Novel c-MYC target genes mediate differential effects on cell proliferation and migration.

EMBO Rep *8*, 70-76.

Carpten, J.D., Faber, A.L., Horn, C., Donoho, G.P., Briggs, S.L., Robbins, C.M., Hostetter, G., Boguslawski, S., Moses, T.Y., Savage, S., *et al.* (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature *448*, 439-444.

Carracedo, A., Baselga, J., and Pandolfi, P.P. (2008a). Deconstructing feedback-signaling networks to improve anticancer therapy with mTORC1 inhibitors. Cell Cycle *7*, 3805-3809.

Carracedo, A., Ma, L., Teruya-Feldstein, J., Rojo, F., Salmena, L., Alimonti, A., Egia, A., Sasaki, A.T., Thomas, G., Kozma, S.C., *et al.* (2008b). Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest *118*, 3065-3074.

Carver, B.S., Chapinski, C., Wongvipat, J., Hieronymus, H., Chen, Y., Chandarlapaty, S., Arora, V.K., Le, C., Koutcher, J., Scher, H., *et al.* (2011). Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. Cancer Cell *19*, 575-586.

Chakrabarty, A., Sanchez, V., Kuba, M.G., Rinehart, C., and Arteaga, C.L. (2011). Feedback upregulation of HER3 (ErbB3) expression and activity attenuates antitumor effect of PI3K inhibitors. Proc Natl Acad Sci U S A.

Chan, S., Scheulen, M.E., Johnston, S., Mross, K., Cardoso, F., Dittrich, C., Eiermann, W., Hess, D., Morant, R., Semiglazov, V., *et al.* (2005). Phase II study of temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer. J Clin Oncol *23*, 5314-5322.

Chan, T.A., Glockner, S., Yi, J.M., Chen, W., Van Neste, L., Cope, L., Herman, J.G., Velculescu, V., Schuebel, K.E., Ahuja, N., *et al.* (2008). Convergence of mutation and epigenetic alterations identifies common genes in cancer that predict for poor prognosis. PLoS Med *5*, e114.

Chandarlapaty, S., Sawai, A., Scaltriti, M., Rodrik-Outmezguine, V., Grbovic-Huezo, O., Serra, V., Majumder, P.K., Baselga, J., and Rosen, N. (2011). AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell *19*, 58-71.

Chen, W., Arroyo, J.D., Timmons, J.C., Possemato, R., and Hahn, W.C. (2005). Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. Cancer Res *65*, 8183-8192.

Chen, W., Possemato, R., Campbell, K.T., Plattner, C.A., Pallas, D.C., and Hahn, W.C. (2004). Identification of specific PP2A complexes involved in human cell transformation. Cancer Cell *5*, 127-136.

Chiarini, F., Grimaldi, C., Ricci, F., Tazzari, P.L., Evangelisti, C., Ognibene, A., Battistelli, M., Falcieri, E., Melchionda, F., Pession, A., *et al.* (2010). Activity of the novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 against T-cell acute lymphoblastic leukemia. Cancer Res *70*, 8097-8107.

Cho, D.C., Cohen, M.B., Panka, D.J., Collins, M., Ghebremichael, M., Atkins, M.B., Signoretti, S., and Mier, J.W. (2010). The efficacy of the novel dual PI3-kinase/mTOR inhibitor NVP-BEZ235 compared with rapamycin in renal cell carcinoma. Clin Cancer Res *16*, 3628-3638.

Crazzolara, R., Bradstock, K.F., and Bendall, L.J. (2009). RAD001 (Everolimus) induces autophagy in acute lymphoblastic leukemia. Autophagy *5*, 727-728.

Cully, M., You, H., Levine, A.J., and Mak, T.W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat Rev Cancer *6*, 184-192.

Dancey, J. (2010). mTOR signaling and drug development in cancer. Nat Rev Clin Oncol *7*, 209-219.

Dilling, M.B., Germain, G.S., Dudkin, L., Jayaraman, A.L., Zhang, X., Harwood, F.C., and Houghton, P.J. (2002). 4E-binding proteins, the suppressors of eukaryotic initiation factor 4E, are down-regulated in cells with acquired or intrinsic resistance to rapamycin. J Biol Chem *277*, 13907-13917.

Ding, L., Getz, G., Wheeler, D.A., Mardis, E.R., McLellan, M.D., Cibulskis, K., Sougnez, C., Greulich, H., Muzny, D.M., Morgan, M.B., *et al.* (2008). Somatic mutations affect key pathways in lung adenocarcinoma. Nature *455*, 1069-1075.

Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E.,

and Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science *314*, 467-471.

Dowling, R.J., Pollak, M., and Sonenberg, N. (2009). Current status and challenges associated with targeting mTOR for cancer therapy. BioDrugs *23*, 77-91.

Dyrskjot, L., Kruhoffer, M., Thykjaer, T., Marcussen, N., Jensen, J.L., Moller, K., and Orntoft, T.F. (2004). Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. Cancer Res *64*, 4040-4048.

Easton, J.B., and Houghton, P.J. (2006). mTOR and cancer therapy. Oncogene *25*, 6436-6446.

Eichhorn, P.J., Creyghton, M.P., and Bernards, R. (2009). Protein phosphatase 2A regulatory subunits and cancer. Biochim Biophys Acta *1795*, 1-15.

Engelman, J.A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer *9*, 550-562.

Engelman, J.A., and Janne, P.A. (2008). Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. Clin Cancer Res *14*, 2895-2899.

Engelman, J.A., Luo, J., and Cantley, L.C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet *7*, 606-619.

Engelman, J.A., Zejnullahu, K., Mitsudomi, T., Song, Y., Hyland, C., Park, J.O., Lindeman, N., Gale, C.M., Zhao, X., Christensen, J.*, et al.* (2007). MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science *316*, 1039-1043.

Ericson, K., Gan, C., Cheong, I., Rago, C., Samuels, Y., Velculescu, V.E., Kinzler, K.W., Huso, D.L., Vogelstein, B., and Papadopoulos, N. (2010). Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation. Proc Natl Acad Sci U S A *107*, 2598-2603.

Esteller, M. (2000). Epigenetic lesions causing genetic lesions in human cancer:

promoter hypermethylation of DNA repair genes. Eur J Cancer *36*, 2294-2300.

Esteller, M. (2008). Epigenetics in cancer. N Engl J Med *358*, 1148-1159.

Esteller, M., Fraga, M.F., Guo, M., Garcia-Foncillas, J., Hedenfalk, I., Godwin, A.K., Trojan, J., Vaurs-Barriere, C., Bignon, Y.J., Ramus, S., *et al.* (2001). DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet *10*, 3001-3007.

Faivre, S., Kroemer, G., and Raymond, E. (2006). Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov *5*, 671-688.

Fan, Q.W., Knight, Z.A., Goldenberg, D.D., Yu, W., Mostov, K.E., Stokoe, D., Shokat, K.M., and Weiss, W.A. (2006). A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. Cancer Cell *9*, 341-349.

Feldman, R.I., Wu, J.M., Polokoff, M.A., Kochanny, M.J., Dinter, H., Zhu, D., Biroc, S.L., Alicke, B., Bryant, J., Yuan, S., *et al.* (2005). Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1. J Biol Chem *280*, 19867-19874.

Futreal, P.A., Coin, L., Marshall, M., Down, T., Hubbard, T., Wooster, R., Rahman, N., and Stratton, M.R. (2004). A census of human cancer genes. Nat Rev Cancer *4*, 177-183.

Gingras, A.C., Raught, B., and Sonenberg, N. (2004). mTOR signaling to translation. Curr Top Microbiol Immunol *279*, 169-197.

Graff, J.R., Konicek, B.W., Carter, J.H., and Marcusson, E.G. (2008). Targeting the eukaryotic translation initiation factor 4E for cancer therapy. Cancer Res *68*, 631-634.

Granville, C.A., Memmott, R.M., Gills, J.J., and Dennis, P.A. (2006). Handicapping the race to develop inhibitors of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway. Clin Cancer Res *12*, 679-689.

Groves, M.R., Hanlon, N., Turowski, P., Hemmings, B.A., and Barford, D. (1999). The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. Cell *96*, 99-110.

Guertin, D.A., and Sabatini, D.M. (2005). An expanding role for mTOR in cancer. Trends Mol Med *11*, 353-361.

Guertin, D.A., and Sabatini, D.M. (2007). Defining the role of mTOR in cancer. Cancer Cell *12*, 9-22.

Guertin, D.A., and Sabatini, D.M. (2009). The pharmacology of mTOR inhibition. Sci Signal *2*, pe24.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464-468.

Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and Weinberg, R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol Cell Biol *22*, 2111-2123.

Hammerman, P.S., Fox, C.J., Birnbaum, M.J., and Thompson, C.B. (2005). Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. Blood *105*, 4477-4483.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev *18*, 1926-1945.

Heinonen, H., Nieminen, A., Saarela, M., Kallioniemi, A., Klefstrom, J., Hautaniemi, S., and Monni, O. (2008). Deciphering downstream gene targets of PI3K/mTOR/p70S6K pathway in breast cancer. BMC Genomics *9*, 348.

Herman, J.G., and Baylin, S.B. (2003). Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med *349*, 2042-2054.

Herman, J.G., Merlo, A., Mao, L., Lapidus, R.G., Issa, J.P., Davidson, N.E., Sidransky, D., and Baylin, S.B. (1995). Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res *55*, 4525-4530.

Herman, S.E., Gordon, A.L., Wagner, A.J., Heerema, N.A., Zhao, W., Flynn, J.M., Jones, J., Andritsos, L., Puri, K.D., Lannutti, B.J., *et al.* (2010). Phosphatidylinositol

3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. Blood *116*, 2078-2088.

Hirai, H., Sootome, H., Nakatsuru, Y., Miyama, K., Taguchi, S., Tsujioka, K., Ueno, Y., Hatch, H., Majumder, P.K., Pan, B.S., *et al.* (2010). MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther *9*, 1956-1967.

Hsieh, A.C., Costa, M., Zollo, O., Davis, C., Feldman, M.E., Testa, J.R., Meyuhas, O., Shokat, K.M., and Ruggero, D. (2010). Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E. Cancer Cell *17*, 249-261.

Hsu, P.P., Kang, S.A., Rameseder, J., Zhang, Y., Ottina, K.A., Lim, D., Peterson, T.R., Choi, Y., Gray, N.S., Yaffe, M.B., *et al.* (2011). The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. Science *332*, 1317-1322.

Hudes, G., Carducci, M., Tomczak, P., Dutcher, J., Figlin, R., Kapoor, A., Staroslawska, E., Sosman, J., McDermott, D., Bodrogi, I., *et al.* (2007). Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. N Engl J Med *356*, 2271-2281.

Ihle, N.T., Paine-Murrieta, G., Berggren, M.I., Baker, A., Tate, W.R., Wipf, P., Abraham, R.T., Kirkpatrick, D.L., and Powis, G. (2005). The phosphatidylinositol-3-kinase inhibitor PX-866 overcomes resistance to the epidermal growth factor receptor inhibitor gefitinib in A-549 human non-small cell lung cancer xenografts. Mol Cancer Ther *4*, 1349-1357.

Ilic, N., Utermark, T., Widlund, H.R., and Roberts, T.M. (2011). PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis. Proc Natl Acad Sci U S A *108*, E699-708.

Janes, M.R., Limon, J.J., So, L., Chen, J., Lim, R.J., Chavez, M.A., Vu, C., Lilly, M.B., Mallya, S., Ong, S.T., *et al.* (2010). Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. Nat Med *16*, 205-213.

Janssens, V., and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J

353, 417-439.

Janssens, V., Goris, J., and Van Hoof, C. (2005). PP2A: the expected tumor suppressor. Curr Opin Genet Dev *15*, 34-41.

Jia, S., Liu, Z., Zhang, S., Liu, P., Zhang, L., Lee, S.H., Zhang, J., Signoretti, S., Loda, M., Roberts, T.M.*, et al.* (2008). Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. Nature *454*, 776-779.

Jiang, X., Tan, J., Li, J., Kivimae, S., Yang, X., Zhuang, L., Lee, P.L., Chan, M.T., Stanton, L.W., Liu, E.T.*, et al.* (2008). DACT3 is an epigenetic regulator of Wnt/beta-catenin signaling in colorectal cancer and is a therapeutic target of histone modifications. Cancer Cell *13*, 529-541.

Jones, P.A., and Baylin, S.B. (2007). The epigenomics of cancer. Cell *128*, 683-692.

Jones, S., Zhang, X., Parsons, D.W., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Kamiyama, H., Jimeno, A.*, et al.* (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science *321*, 1801-1806.

Junttila, M.R., Puustinen, P., Niemela, M., Ahola, R., Arnold, H., Bottzauw, T., Ala-aho, R., Nielsen, C., Ivaska, J., Taya, Y.*, et al.* (2007). CIP2A inhibits PP2A in human malignancies. Cell *130*, 51-62.

Junttila, T.T., Akita, R.W., Parsons, K., Fields, C., Lewis Phillips, G.D., Friedman, L.S., Sampath, D., and Sliwkowski, M.X. (2009). Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell *15*, 429-440.

Kan, Z., Jaiswal, B.S., Stinson, J., Janakiraman, V., Bhatt, D., Stern, H.M., Yue, P., Haverty, P.M., Bourgon, R., Zheng, J.*, et al.* (2010). Diverse somatic mutation patterns and pathway alterations in human cancers. Nature *466*, 869-873.

Kentsis, A., Topisirovic, I., Culjkovic, B., Shao, L., and Borden, K.L. (2004). Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap. Proc Natl Acad Sci U S A *101*, 18105-18110.

Kikani, C.K., Dong, L.Q., and Liu, F. (2005). "New"-clear functions of PDK1:

beyond a master kinase in the cytosol? J Cell Biochem *96*, 1157-1162.

Kim, J., Woo, A.J., Chu, J., Snow, J.W., Fujiwara, Y., Kim, C.G., Cantor, A.B., and Orkin, S.H. (2010). A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell *143*, 313-324.

Kinzler, K.W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature *386*, 761, 763.

Knight, Z.A., Gonzalez, B., Feldman, M.E., Zunder, E.R., Goldenberg, D.D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., *et al.* (2006). A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. Cell *125*, 733-747.

Kobayashi, S., Boggon, T.J., Dayaram, T., Janne, P.A., Kocher, O., Meyerson, M., Johnson, B.E., Eck, M.J., Tenen, D.G., and Halmos, B. (2005). EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med *352*, 786-792.

Kondo, Y., Shen, L., Cheng, A.S., Ahmed, S., Boumber, Y., Charo, C., Yamochi, T., Urano, T., Furukawa, K., Kwabi-Addo, B., *et al.* (2008). Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet *40*, 741-750.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275*, 1784-1787.

Kuo, Y.C., Huang, K.Y., Yang, C.H., Yang, Y.S., Lee, W.Y., and Chiang, C.W. (2008). Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. J Biol Chem *283*, 1882-1892.

Li, D., Shimamura, T., Ji, H., Chen, L., Haringsma, H.J., McNamara, K., Liang, M.C., Perera, S.A., Zaghlul, S., Borgman, C.L., *et al.* (2007). Bronchial and peripheral murine lung carcinomas induced by T790M-L858R mutant EGFR respond to HKI-272 and rapamycin combination therapy. Cancer Cell *12*, 81-93.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., *et al.* (1997). PTEN, a putative protein tyrosine

phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943-1947.

Lin, H.J., Hsieh, F.C., Song, H., and Lin, J. (2005). Elevated phosphorylation and activation of PDK-1/AKT pathway in human breast cancer. Br J Cancer *93*, 1372-1381.

Liu, P., Cheng, H., Roberts, T.M., and Zhao, J.J. (2009). Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov *8*, 627-644.

Liu, P., Cheng, H., Santiago, S., Raeder, M., Zhang, F., Isabella, A., Yang, J., Semaan, D.J., Chen, C., Fox, E.A., *et al.* (2011). Oncogenic PIK3CA-driven mammary tumors frequently recur via PI3K pathway-dependent and PI3K pathway-independent mechanisms. Nat Med *17*, 1116-1120.

Longin, S., Zwaenepoel, K., Louis, J.V., Dilworth, S., Goris, J., and Janssens, V. (2007). Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit. J Biol Chem *282*, 26971-26980.

Lung, H.L., Bangarusamy, D.K., Xie, D., Cheung, A.K., Cheng, Y., Kumaran, M.K., Miller, L., Liu, E.T., Guan, X.Y., Sham, J.S., *et al.* (2005). THY1 is a candidate tumour suppressor gene with decreased expression in metastatic nasopharyngeal carcinoma. Oncogene *24*, 6525-6532.

Maira, S.M., Stauffer, F., Brueggen, J., Furet, P., Schnell, C., Fritsch, C., Brachmann, S., Chene, P., De Pover, A., Schoemaker, K., *et al.* (2008). Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther *7*, 1851-1863.

Majumder, P.K., Febbo, P.G., Bikoff, R., Berger, R., Xue, Q., McMahon, L.M., Manola, J., Brugarolas, J., McDonnell, T.J., Golub, T.R., *et al.* (2004). mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med *10*, 594-601.

Mao, J.H., Kim, I.J., Wu, D., Climent, J., Kang, H.C., DelRosario, R., and Balmain, A. (2008). FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science *321*, 1499-1502.

Markman, B., Dienstmann, R., and Tabernero, J. (2010). Targeting the PI3K/Akt/mTOR pathway--beyond rapalogs. Oncotarget *1*, 530-543.

Martinez Hoyos, J., Ferraro, A., Sacchetti, S., Keller, S., De Martino, I., Borbone, E., Pallante, P., Fedele, M., Montanaro, D., Esposito, F.*, et al.* (2009). HAND1 gene expression is negatively regulated by the High Mobility Group A1 proteins and is drastically reduced in human thyroid carcinomas. Oncogene *28*, 876-885.

Maurer, M., Su, T., Saal, L.H., Koujak, S., Hopkins, B.D., Barkley, C.R., Wu, J., Nandula, S., Dutta, B., Xie, Y.*, et al.* (2009). 3-Phosphoinositide-dependent kinase 1 potentiates upstream lesions on the phosphatidylinositol 3-kinase pathway in breast carcinoma. Cancer Res *69*, 6299-6306.

McGarvey, K.M., Van Neste, L., Cope, L., Ohm, J.E., Herman, J.G., Van Criekinge, W., Schuebel, K.E., and Baylin, S.B. (2008). Defining a chromatin pattern that characterizes DNA-hypermethylated genes in colon cancer cells. Cancer Res *68*, 5753-5759.

Meissner, A., Mikkelsen, T.S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B.E., Nusbaum, C., Jaffe, D.B.*, et al.* (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature *454*, 766-770.

Meric-Bernstam, F., and Gonzalez-Angulo, A.M. (2009). Targeting the mTOR signaling network for cancer therapy. J Clin Oncol *27*, 2278-2287.

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B., and Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med *1*, 686-692.

Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, E.S., and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature *454*, 49-55.

Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T.K., Koche, R.P.*, et al.* (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553-560.

Mora, A., Komander, D., van Aalten, D.M., and Alessi, D.R. (2004). PDK1, the master regulator of AGC kinase signal transduction. Semin Cell Dev Biol *15*, 161-170.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science *275*, 1787-1790.

Mumby, M. (2007). PP2A: unveiling a reluctant tumor suppressor. Cell *130*, 21-24.

Nelsen, C.J., Rickheim, D.G., Tucker, M.M., Hansen, L.K., and Albrecht, J.H. (2003). Evidence that cyclin D1 mediates both growth and proliferation downstream of TOR in hepatocytes. J Biol Chem *278*, 3656-3663.

Neviani, P., Santhanam, R., Trotta, R., Notari, M., Blaser, B.W., Liu, S., Mao, H., Chang, J.S., Galietta, A., Uttam, A.*, et al.* (2005). The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell *8*, 355-368.

O'Reilly, K.E., Rojo, F., She, Q.B., Solit, D., Mills, G.B., Smith, D., Lane, H., Hofmann, F., Hicklin, D.J., Ludwig, D.L.*, et al.* (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res *66*, 1500-1508.

Ohm, J.E., McGarvey, K.M., Yu, X., Cheng, L., Schuebel, K.E., Cope, L., Mohammad, H.P., Chen, W., Daniel, V.C., Yu, W.*, et al.* (2007). A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet *39*, 237-242.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Okochi-Takada, E., Nakazawa, K., Wakabayashi, M., Mori, A., Ichimura, S., Yasugi, T., and Ushijima, T. (2006). Silencing of the UCHL1 gene in human colorectal and ovarian cancers. Int J Cancer *119*, 1338-1344.

Ooi, S.K., Qiu, C., Bernstein, E., Li, K., Jia, D., Yang, Z., Erdjument-Bromage, H., Tempst, P., Lin, S.P., Allis, C.D.*, et al.* (2007). DNMT3L connects unmethylated

lysine 4 of histone H3 to de novo methylation of DNA. Nature *448*, 714-717.

Padmanabhan, S., Mukhopadhyay, A., Narasimhan, S.D., Tesz, G., Czech, M.P., and Tissenbaum, H.A. (2009). A PP2A regulatory subunit regulates C. elegans insulin/IGF-1 signaling by modulating AKT-1 phosphorylation. Cell *136*, 939-951.

Pallas, D.C., Shahrik, L.K., Martin, B.L., Jaspers, S., Miller, T.B., Brautigan, D.L., and Roberts, T.M. (1990). Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell *60*, 167-176.

Pao, W., Miller, V.A., Politi, K.A., Riely, G.J., Somwar, R., Zakowski, M.F., Kris, M.G., and Varmus, H. (2005). Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med *2*, e73.

Peifer, C., and Alessi, D.R. (2008). Small-molecule inhibitors of PDK1. ChemMedChem *3*, 1810-1838.

Ponder, B.A. (2001). Cancer genetics. Nature *411*, 336-341.

Pouyssegur, J., Dayan, F., and Mazure, N.M. (2006). Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature *441*, 437-443.

Rhee, I., Bachman, K.E., Park, B.H., Jair, K.W., Yen, R.W., Schuebel, K.E., Cui, H., Feinberg, A.P., Lengauer, C., Kinzler, K.W.*, et al.* (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature *416*, 552-556.

Rhodes, D.R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A., and Chinnaiyan, A.M. (2004a). Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression. Proc Natl Acad Sci U S A *101*, 9309-9314.

Rhodes, D.R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A., and Chinnaiyan, A.M. (2004b). ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia *6*, 1-6.

Rhodes, N., Heerding, D.A., Duckett, D.R., Eberwein, D.J., Knick, V.B., Lansing, T.J., McConnell, R.T., Gilmer, T.M., Zhang, S.Y., Robell, K.*, et al.* (2008). Characterization of an Akt kinase inhibitor with potent pharmacodynamic and

antitumor activity. Cancer Res *68*, 2366-2374.

Ruediger, R., Fields, K., and Walter, G. (1999). Binding specificity of protein phosphatase 2A core enzyme for regulatory B subunits and T antigens. J Virol *73*, 839-842.

Sabatini, D.M. (2006). mTOR and cancer: insights into a complex relationship. Nat Rev Cancer *6*, 729-734.

Sablina, A.A., Chen, W., Arroyo, J.D., Corral, L., Hector, M., Bulmer, S.E., DeCaprio, J.A., and Hahn, W.C. (2007). The tumor suppressor PP2A Abeta regulates the RalA GTPase. Cell *129*, 969-982.

Sablina, A.A., Hector, M., Colpaert, N., and Hahn, W.C. (2010). Identification of PP2A complexes and pathways involved in cell transformation. Cancer Res *70*, 10474-10484.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S.M., Riggins, G.J., *et al.* (2004). High frequency of mutations of the PIK3CA gene in human cancers. Science *304*, 554.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell *25*, 903-915.

Sanchez-Carbayo, M., Socci, N.D., Lozano, J., Saint, F., and Cordon-Cardo, C. (2006). Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. J Clin Oncol *24*, 778-789.

Sansom, O.J., Meniel, V.S., Muncan, V., Phesse, T.J., Wilkins, J.A., Reed, K.R., Vass, J.K., Athineos, D., Clevers, H., and Clarke, A.R. (2007). Myc deletion rescues Apc deficiency in the small intestine. Nature *446*, 676-679.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol *14*, 1296-1302.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F.,

Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell *22*, 159-168.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Sawyers, C.L. (2008). The cancer biomarker problem. Nature *452*, 548-552.

Schuebel, K.E., Chen, W., Cope, L., Glockner, S.C., Suzuki, H., Yi, J.M., Chan, T.A., Van Neste, L., Van Criekinge, W., van den Bosch, S.*, et al.* (2007). Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. PLoS Genet *3*, 1709-1723.

Scott, K.L., Kabbarah, O., Liang, M.C., Ivanova, E., Anagnostou, V., Wu, J., Dhakal, S., Wu, M., Chen, S., Feinberg, T.*, et al.* (2009). GOLPH3 modulates mTOR signalling and rapamycin sensitivity in cancer. Nature *459*, 1085-1090.

Seeling, J.M., Miller, J.R., Gil, R., Moon, R.T., White, R., and Virshup, D.M. (1999). Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science *283*, 2089-2091.

Sekulic, A., Hudson, C.C., Homme, J.L., Yin, P., Otterness, D.M., Karnitz, L.M., and Abraham, R.T. (2000). A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res *60*, 3504-3513.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. Nat Rev Cancer *3*, 721-732.

Serra, V., Markman, B., Scaltriti, M., Eichhorn, P.J., Valero, V., Guzman, M., Botero, M.L., Llonch, E., Atzori, F., Di Cosimo, S.*, et al.* (2008). NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res *68*, 8022-8030.

She, Q.B., Chandarlapaty, S., Ye, Q., Lobo, J., Haskell, K.M., Leander, K.R., DeFeo-Jones, D., Huber, H.E., and Rosen, N. (2008). Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. PLoS One *3*, e3065.

Sjoblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N., *et al.* (2006). The consensus coding sequences of human breast and colorectal cancers. Science *314*, 268-274.

Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993). The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. Cell *75*, 887-897.

Stommel, J.M., Kimmelman, A.C., Ying, H., Nabioullin, R., Ponugoti, A.H., Wiedemeyer, R., Stegh, A.H., Bradner, J.E., Ligon, K.L., Brennan, C., *et al.* (2007). Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. Science *318*, 287-290.

Stone, S.R., Hofsteenge, J., and Hemmings, B.A. (1987). Molecular cloning of cDNAs encoding two isoforms of the catalytic subunit of protein phosphatase 2A. Biochemistry *26*, 7215-7220.

Stratton, M.R., Campbell, P.J., and Futreal, P.A. (2009). The cancer genome. Nature *458*, 719-724.

Su, L.K., Vogelstein, B., and Kinzler, K.W. (1993). Association of the APC tumor suppressor protein with catenins. Science *262*, 1734-1737.

Su, Y., Fu, C., Ishikawa, S., Stella, A., Kojima, M., Shitoh, K., Schreiber, E.M., Day, B.W., and Liu, B. (2008). APC is essential for targeting phosphorylated beta-catenin to the SCFbeta-TrCP ubiquitin ligase. Mol Cell *32*, 652-661.

Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. (1988). Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. Proc Natl Acad Sci U S A *85*, 1768-1771.

Suzuki, H., Gabrielson, E., Chen, W., Anbazhagan, R., van Engeland, M., Weijenberg, M.P., Herman, J.G., and Baylin, S.B. (2002). A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet *31*, 141-149.

Suzuki, H., Watkins, D.N., Jair, K.W., Schuebel, K.E., Markowitz, S.D., Chen, W.D., Pretlow, T.P., Yang, B., Akiyama, Y., Van Engeland, M., *et al.* (2004). Epigenetic

inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet *36*, 417-422.

Tellez, C.S., Shen, L., Estecio, M.R., Jelinek, J., Gershenwald, J.E., and Issa, J.P. (2009). CpG island methylation profiling in human melanoma cell lines. Melanoma Res *19*, 146-155.

Thomas, G.V., Tran, C., Mellinghoff, I.K., Welsbie, D.S., Chan, E., Fueger, B., Czernin, J., and Sawyers, C.L. (2006). Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat Med *12*, 122-127.

Vasudevan, K.M., Barbie, D.A., Davies, M.A., Rabinovsky, R., McNear, C.J., Kim, J.J., Hennessy, B.T., Tseng, H., Pochanard, P., Kim, S.Y.*, et al.* (2009). AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. Cancer Cell *16*, 21-32.

Virshup, D.M., and Shenolikar, S. (2009). From promiscuity to precision: protein phosphatases get a makeover. Mol Cell *33*, 537-545.

Vivanco, I., and Sawyers, C.L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer *2*, 489-501.

Wang, S.S., Esplin, E.D., Li, J.L., Huang, L., Gazdar, A., Minna, J., and Evans, G.A. (1998). Alterations of the PPP2R1B gene in human lung and colon cancer. Science *282*, 284-287.

Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M., and Schubeler, D. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet *39*, 457-466.

Weinstein, I.B. (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science *297*, 63-64.

Welcker, M., and Clurman, B.E. (2008). FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer *8*, 83-93.

Westermarck, J., and Hahn, W.C. (2008). Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med *14*, 152-160.

Witzig, T.E., Geyer, S.M., Ghobrial, I., Inwards, D.J., Fonseca, R., Kurtin, P., Ansell, S.M., Luyun, R., Flynn, P.J., Morton, R.F., *et al.* (2005). Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma. J Clin Oncol *23*, 5347-5356.

Wong, D.J., Liu, H., Ridky, T.W., Cassarino, D., Segal, E., and Chang, H.Y. (2008). Module map of stem cell genes guides creation of epithelial cancer stem cells. Cell Stem Cell *2*, 333-344.

Wong, K.K., Engelman, J.A., and Cantley, L.C. (2010). Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev *20*, 87-90.

Wood, L.D., Parsons, D.W., Jones, S., Lin, J., Sjoblom, T., Leary, R.J., Shen, D., Boca, S.M., Barber, T., Ptak, J., *et al.* (2007). The genomic landscapes of human breast and colorectal cancers. Science *318*, 1108-1113.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.

Xing, Y., Li, Z., Chen, Y., Stock, J.B., Jeffrey, P.D., and Shi, Y. (2008). Structural mechanism of demethylation and inactivation of protein phosphatase 2A. Cell *133*, 154-163.

Yeh, E., Cunningham, M., Arnold, H., Chasse, D., Monteith, T., Ivaldi, G., Hahn, W.C., Stukenberg, P.T., Shenolikar, S., Uchida, T., *et al.* (2004). A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat Cell Biol *6*, 308-318.

Yi, J.M., Dhir, M., Van Neste, L., Downing, S.R., Jeschke, J., Glockner, S.C., de Freitas Calmon, M., Hooker, C.M., Funes, J.M., Boshoff, C., *et al.* (2011). Genomic and epigenomic integration identifies a prognostic signature in colon cancer. Clin Cancer Res *17*, 1535-1545.

Yu, J., Cao, Q., Mehra, R., Laxman, B., Tomlins, S.A., Creighton, C.J., Dhanasekaran, S.M., Shen, R., Chen, G., Morris, D.S., *et al.* (2007). Integrative genomics analysis reveals silencing of beta-adrenergic signaling by polycomb in prostate cancer. Cancer Cell *12*, 419-431.

Yu, Y., Yoon, S.O., Poulogiannis, G., Yang, Q., Ma, X.M., Villen, J., Kubica, N.,

Hoffman, G.R., Cantley, L.C., Gygi, S.P., *et al.* (2011). Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. Science *332*, 1322-1326.

Yue, W., Sun, Q., Dacic, S., Landreneau, R.J., Siegfried, J.M., Yu, J., and Zhang, L. (2008). Downregulation of Dkk3 activates beta-catenin/TCF-4 signaling in lung cancer. Carcinogenesis *29*, 84-92.

Yun, C.H., Boggon, T.J., Li, Y., Woo, M.S., Greulich, H., Meyerson, M., and Eck, M.J. (2007). Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. Cancer Cell *11*, 217-227.

Zhang, W., Glockner, S.C., Guo, M., Machida, E.O., Wang, D.H., Easwaran, H., Van Neste, L., Herman, J.G., Schuebel, K.E., Watkins, D.N., *et al.* (2008). Epigenetic inactivation of the canonical Wnt antagonist SRY-box containing gene 17 in colorectal cancer. Cancer Res *68*, 2764-2772.

Zhao, J.J., Cheng, H., Jia, S., Wang, L., Gjoerup, O.V., Mikami, A., and Roberts, T.M. (2006). The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. Proc Natl Acad Sci U S A *103*, 16296-16300.

Zhao, J.J., Gjoerup, O.V., Subramanian, R.R., Cheng, Y., Chen, W., Roberts, T.M., and Hahn, W.C. (2003). Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell *3*, 483-495.

Zhao, X.D., Han, X., Chew, J.L., Liu, J., Chiu, K.P., Choo, A., Orlov, Y.L., Sung, W.K., Shahab, A., Kuznetsov, V.A., *et al.* (2007). Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell *1*, 286-298.

Zunder, E.R., Knight, Z.A., Houseman, B.T., Apsel, B., and Shokat, K.M. (2008). Discovery of drug-resistant and drug-sensitizing mutations in the oncogenic PI3K isoform p110 alpha. Cancer Cell *14*, 180-192.

List of Publications

1. Tan Jing, Yu Q. PDK1-driven Myc signaling regulates cellular response to mTOR inhibitors. Cell Cycle. 2011 Apr 1;10(7):1019-20. Epub 2011 Apr 1. PMID: 21430441.

2. Tan Jing, Lee PL, Li Z, Jiang X, Lim YC, Hooi SC, Yu Q. B55β-associated PP2A complex controls PDK1-directed myc signaling and modulates rapamycin sensitivity in colorectal cancer. Cancer Cell. 2010 Nov 16;18 (5):459-71. PMID: 21075311.

3. Jiang X*, Tan Jing*, Li J, Kivimäe S, Yang X, Zhuang L, Lee PL, Chan MT, Stanton LW, Liu ET, Cheyette BN, Yu Q. DACT3 is an epigenetic regulator of Wnt/beta-catenin signaling in colorectal cancer and is a therapeutic target of histone modifications. Cancer Cell. 2008 Jun;13(6):529-41. PubMed PMID: 18538736 (* JX and TJ contributed equally).