Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Jennifer B. Jackson Date

Investigating the Tumor Suppressive Role of PP2Ac Methylation:
Implications for SV40 and Polyomavirus-mediated Transformation

By

Jennifer B. Jackson
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Biochemistry, Cell, and Developmental Biology

_____
David C. Pallas, Ph.D.
Advisor

_____
Anita Corbett, Ph.D.
Committee Member

_____
Richard Cummings, Ph.D.
Committee Member

_____
Carlos Moreno, Ph.D.
Committee Member

_____
Paula Vertino, Ph.D.
Committee Member

_____
Wei Zhou, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

_____
Date

Investigating the Tumor Suppressive Role of PP2Ac Methylation: Implications for SV40 and Polyomavirus-mediated Transformation

By

Jennifer B. Jackson
B.S., University of Florida, 2002

Advisor: David C. Pallas, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the James T.
Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Program in Biochemistry, Cell, and Developmental Biology
Graduate Division of Biological and Biomedical Sciences
2012

PP2A regulates growth and survival pathways and its dysfunction has been linked to cancer. The heterotrimeric PP2A holoenzyme consists of catalytic C (PP2Ac), structural A, and regulatory B-type subunits. Reversible methylation of PP2Ac by LCMT-1 and PME-1 differentially regulates the binding of certain B-type subunits and thus PP2A function. In addition to the cellular B-type subunits, some DNA tumor virus oncoproteins including polyomavirus small (PyST) and middle (PyMT) tumor antigens and SV40 small tumor antigen (SVST) function as viral B-type subunits. These viral oncoproteins alter PP2A function and promote transformation in part by replacing certain cellular B-type subunits. Interestingly, while the B-type subunits replaced by these oncoproteins appear to exhibit a binding preference for A/C dimers containing methylated PP2Ac (methylation-sensitive), the only tumor antigen tested to date, PyMT, does not (methylation-insensitive). In Chapter 3.1, I hypothesized that circumventing the cellular control of PP2A by PP2Ac methylation is a general strategy for MT- and ST-mediated transformation. Results indicated that SVST and PyST also bind to PP2A in a methylation-insensitive manner. Furthermore, reduction of PP2Ac methylation enhanced transformation through activation of the Akt and S6K1 pathways. These results support the hypothesis that replacing methylation-sensitive B-type subunits with methylation-insensitive B-type subunits, thereby circumventing control of PP2A by methylation, is a general strategy for MT and ST-mediated transformation. In Chapter 3.2, I tested the individual contributions to transformation of the N-terminal, PP2A-independent portion of SVST and of reducing methylation-sensitive PP2A complexes in conjunction with B'γ knockdown. The findings revealed that MT- and ST-mediated transformation is likely modulated through the concomitant expression of the N-terminal domain and reduction of multiple methylation-sensitive PP2A complexes. Finally, in Chapter 3.3, I found that reducing PME-1 could reduce transformation. Overall, these studies provide insights into a novel strategy that T antigens may utilize to circumvent cellular regulation of PP2A by methylation. The research here also provides evidence for the involvement of PP2Ac methylation in supporting the tumor suppressive role of PP2A. Therefore, disruption of PP2Ac methylation may contribute to cancer and promoting this methylation (e.g. PME-1 inhibitors) may serve as a therapeutic target for cancer.

Investigating the Tumor Suppressive Role of PP2Ac Methylation: Implications for SV40 and Polyomavirus-mediated Transformation

By

Jennifer B. Jackson
B.S., University of Florida, 2002

Advisor: David C. Pallas, Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Program in Biochemistry, Cell, and Developmental Biology
Graduate Division of Biological and Biomedical Sciences
2012

Acknowledgements

It is a great pleasure to thank everyone who helped me throughout my graduate school career. First and foremost, I am sincerely grateful for the my advisor, David Pallas, who has provided me with endless support, patience, and guidance throughout my stay in his lab. He has instilled in me the ability to explore novel ideas, navigate through setbacks (both personal and professional), and critically think about the "big picture". Without his feedback, encouragement, and scientific expertise, my successful transition from student to scientist would not have been possible. I would also like to recognize and heartily thank my committee members, Anita Corbett, Richard Cummings, Carlos Moreno, Paula Vertino, and Wei Zhou, for their valuable time and feedback throughout the course of graduate school. Their invaluable expertise has guided me through several difficult roadblocks in my research and has provided me with the confidence to move forward to the next chapter of my scientific career. I would like to thank the past and present members of the Pallas lab, especially Jocelyn Lee, Quiana Kern, Jessie Hwang, and Kyung Park, for assisting me in the lab and for being supportive friends and colleagues. Finally, I would like to recognize Emory University and the BCDB program for taking a chance on me and providing me with the opportunity to study at an exceptional institution.

I would like to express my gratitude for my good friends, Maria Dawson, Sandii Brimble, Lisa Nelson, Briana Madden and Katie Wall, for always being there for me during the good and tough times and for reminding me, when needed, to mix in some downtime during my studies. I would also like to recognize my brothers and sisters, Bo, Tracey, Bonnie and Jerry for their unwavering support throughout my life and career. Their positive attitudes have always given me to the strength to keep going. Most importantly, I would like to express my deepest gratitude for my parents, Lee and Colleen Jackson, who have never doubted my capabilities or dedication. Their unwavering love, support, and encouragement during graduate school and throughout life have been a blessing and they have always given life to my dreams. Finally, I would like to recognize my late grandparents, Leslyn Keie, and John and Therese Maguire, and dedicate this dissertation to them as they were some of my biggest supporters.

TABLE OF CONTENTS

Page

CHAPTER 1: GENERAL INTRODUCTION 1

Protein Phosphorylation 1

Basic Structure of PP2A 2

Reversible Methylation of PP2A C Subunit 5

Role of PP2Ac Methylation 7

PP2A Function: A Pleotrophic Protein 10

Akt/PKB Pathway 12

Akt-mTOR-S6 Kinase Pathway 14

ERK/MAPK Pathway 17

Cell Cycle 18

Apoptosis 22

Cancer and PP2A 26

PP2A: Evidence for a Tumor Suppressor 27

DNA Tumor Viruses: Targeting PP2A Assembly 30

Simian Virus 40 (SV40) 31

HEKTERASB56γ: A Genetically Defined Transformation Cell Line 34

Polyomavirus 37

Goals of the Dissertation 40

CHAPTER 2: METHODS AND MATERIALS 44

Cell Culture 44

Transfection of shRNAs for Lentivirus Production 44

Lentiviral Titering and Infections 45

Creation of the LCMT-1-Rescue cDNA Sequence 47

Creation of HEKTERASB56γ Cells That Stably Express Akt-AA, PME-1, ST110, or an shRNA-resistant LCMT-1 47

Antibodies 48

Cell Lysis and Western Analysis 49

Transfection Procedure for Expression of SVST, PyST and PP2Ac 50

Immunoprecipitation 50
PP2A C Subunit Methylation Assay 51
Anchorage-Independent Growth (Soft Agar Analysis) 52
Suspension Cultures 53
Anchorage-Dependent Growth Assays 54
Anchorage-Dependent Death Assays 54
Time-Lapse Microscopy 55

CHAPTER 3: RESULTS 56
Chapter 3.1: Circumventing Cellular Control of PP2A by Methylation Promotes Transformation in an Akt-dependent Manner 56
SVST and PyST Do Not Require PP2Ac Methylation for PP2A Heterotrimer Formation 56
Loss of LCMT-1 Promotes Transformation 57
LCMT-1 Knockdown Does Not Affect Cell Size, Proliferation Rate, Survival, or the Akt and p70/p85 S6K Signaling Pathways during Normal Anchorage-Dependent Growth 59
LCMT-1 Knockdown Activates the Akt and p70/p85 S6K Pathways in Anchorage-Independent Conditions 60
Effects of LCMT-1 Knockdown on Growth and Signaling Are Rescued by Exogenous Expression of LCMT-1 63
Akt Activation Is Necessary for the Enhanced Transformation Caused by LCMT-1 Knockdown 65
Overexpression of the PP2Ac Methylesterase, PME-1, Causes Similar Changes in Anchorage-Independent Growth and Signaling As LCMT-1 Knockdown 66

Chapter 3.2: Investigating the Potential Targets of SV40 Small T Antigen and the Potential Contributions of the SV40 Small T Antigen PP2A-independent Region to Transformation 90
Expression of the PP2A-independent region of SVST does not complement B'γ knockdown to further enhance transformation 90

Suppression of methylation-sensitive, SVST-targeted PP2A B-type subunits in combination with B'γ knockdown reveals differential effects on cell growth, survival, and transformation 91
Suppressing Bα 92
Suppressing B'α 93
Suppressing Bδ 95

Chapter 3.3: Sustained PP2Ac Methylation Enhances the Tumor Suppressive Function of PP2A through Down-regulation of the Akt Pathway 109
Suppression of PME-1 Reduces Transformation 109
PME-1 Knockdown Does Not Affect Proliferation Rate or the Akt and p70/p85 S6K Signaling Pathways during Normal Anchorage-Dependent Growth 111
PME-1 Knockdown Downregulates the Akt and p70/p85 S6K Pathways in Anchorage-Independent Conditions 112

CHAPTER 4: DISCUSSION 119
Circumventing Normal Cellular Control of PP2A by PP2Ac Methylation is a General Strategy for ST- and MT-mediated Transformation 119
Expression of the PP2A-independent Region of SVST Does Not Complement B'γ Knockdown in Transformation 125
Suppression of Methylation-Sensitive, SVST-targeted PP2A B-type Subunits in Combination with B'γ Knockdown Reveals Differential Effects on Cell Growth, Survival, and Transformation 127
Sustained PP2Ac Methylation Enhances the Tumor Suppressive Function of PP2A through Down-regulation of the Akt Pathway 137

Closing Remarks: Implications for Cancer 140
Future Directions 143

CHAPTER 5: REFERENCES 149

LIST OF FIGURES

Figure		Page
1.	General Schematic of PP2A Holoenzyme Composition	4
2.	General Schematic of Reversible Methylation of PP2Ac	6
3.	General Schematic of PP2A Functions	11
4.	The HEKTER Experimental Cell Model	35
5.	General model illustrating the regulation of PP2A complexes by PP2Ac methylation and PP2A's regulation of progrowth, prosurvival pathways	41
6.	Incorporation of SVST and PyST into PP2A heterotrimers independently of PP2Ac carboxyl methylation	69
7.	Knocking down LCMT-1 promotes transformation	71
8.	Adherent control and LCMT-1 knockdown lines show no difference in growth, death, or Akt and S6K signaling	73
9.	LCMT-1 knockdown activates Akt and S6K signaling in anchorage-independent conditions	75
10.	Effects of LCMT-1 knockdown on transformation are rescued by expression of an shRNA-resistant LCMT-1	77
11.	LCMT-1 overexpression confirms the tumor suppressive role of PP2Ac methylation	79

Figure Page

12. Effects of LCMT-1 knockdown on biochemical signaling are rescued by re-expression of LCMT-1 81

13. Akt activation is necessary for the enhanced transformation caused by LCMT-1 knockdown 83

14. Effects of LCMT-1 knockdown on biochemical signaling are reversed by expression of dominant-negative Akt 84

15. Overexpression of the PP2A methylesterase, PME-1, enhances transformation 86

16. Overexpression of the PP2A methylesterase, PME-1, elicits similar biochemical changes as LCMT-1 suppression 88

17. Expression of the PP2A-independent region of SVST does not complement B'γ knockdown to further enhance transformation 99

18. Knocking down Bα does not significantly enhance transformation 101

19. Knocking down B'α reduces transformation 103

20. Knocking down Bδ in HeLa cells causes widespread death 105

21. Bδ knockdown or SV40 small T (SVST) expression in HeLa cells causes mitotic defects and apoptosis 107

22. Knocking down PME-1 reduces transformation 114

Figure Page

23. Adherent control and PME-1 knockdown lines show no difference in growth or Akt and S6K signaling 116

24. PME-1 knockdown downregulates the Akt and S6K pathways in anchorage-independent conditions 117

25. Proposed model for the negative regulation of anchorage-independent growth and survival by methylation-sensitive PP2A heterotrimers 145

26. Proposed model for the strategies exploited by SV40 small T (SVST) to promote transformation 147

LIST OF TABLES

Table Page

1. List of shRNAs (acquired from The RNAi Consortium (TRC)) targeting PP2A B-type subunits, LCMT-1 and PME-1 45

CHAPTER 1

General Introduction

Protein Phosphorylation

Reversible phosphorylation is one of the most common post-translational protein modifications in the cell which can regulate numerous cellular processes [1]. Proteins in mammalian cells are typically phosphorylated on tyrosine, serine, and/or threonine residues and this modification can alter the stability, conformation, localization, binding specificity, or activity of the protein [1-3]. Protein phosphorylation and dephosphorylation are catalyzed by protein kinases and protein phosphatases, respectively, and the cell must precisely control the dynamic balance of these enzymes in order to properly regulate cellular function and pathways [4, 5]. In addition, phosphorylation can also be negatively regulated indirectly by other post-translational modifications, including O-GlcNac and acetylation, which compete with phosphorylation at the same residues [6, 7]. Dysregulation of protein phosphorylation events can severely impact normal cell function, consequently leading to multiple diseases including cancer [1, 8-10]; therefore, understanding this regulatory mechanism is critical for developing therapeutic strategies. As a result of the human genome being decoded, it was determined that human DNA encodes over 500 kinases [11]. In contrast, far fewer phosphatases (less than 150) have been revealed [12]. The vast difference in the number of kinases and phosphatases indicate that some phosphatases must function in multiple pathways, by association with regulatory subunits, in order to oppose more than one kinase to regulate cellular homeostasis [13].

Protein phosphatases are generally classified into 3 main families based on the residues they dephosphorylate: the protein serine/threonine phosphatases (PSTP), the protein tyrosine phosphatase (PTP) and the dual specificity phosphatases (DSP) which can dephosphorylate serine, threonine and tyrosine residues [1]. The PSTP family, which consists of PP1, PP2A, PP2B, PP2C, PP4, PP5, PP6, and PP7, accounts for the majority of phosphatase activity in mammalian cells for the reason that 98% of protein phosphorylation occurs on serine and threonine residues [2]. Of the PSTP family members, PP1 and PP2A account for >90% of serine/threonine phosphatase activity in the eukaryotic cell [9]. Furthermore, PP2A is the most abundant phosphatase, constituting approximately 1% of total protein in eukaryotic cells [9, 14, 15], and therefore, plays a crucial role in cellular homeostasis. While a great deal of research has concentrated on the therapeutic importance of kinases in disease models, the significance of modulating cellular signaling and cell function via phosphatases and their potential impact on diseases such as cancer have only begun to be explored.

Basic Structure of PP2A

Protein phosphatase 2A (PP2A), a major serine/threonine phosphatase, is predominantly a heterotrimeric protein which is ubiquitously expressed in eukaryotic cells [9, 16, 17]. PP2A typically consists of a structural/scaffolding A/PR65 subunit, a catalytic C subunit (PP2Ac), and one of the various regulatory B-type subunit (Figure 1), although a small fraction of PP2A does exist as heterodimeric complexes consisting of the structural A and catalytic C subunits or as complexes of the C subunit and Tap42/α4 [18, 19]. In mammalian cells, the A subunit is composed of 15 tandem HEAT

(**H**untingtin-**E**longation-**A** subunit-**T**OR) repeats which can bind to both the regulatory B and catalytic C subunits to bring the two subunits in close proximity to one another. The A subunit exists as α and β isoforms with Aα being the more abundantly expressed form. Although the Aα and Aβ isoforms share 86% sequence identity [20], they are not completely redundant and interestingly, Aβ cannot substitute for loss of Aα [9]. The PP2A C subunit, which contains the phosphatase activity, also exists as α and β isoforms that share 97% homology. The Cα isoform is more abundantly expressed and loss of the Cα gene results in lethality in the murine model system [21]. The PP2A C subunit (PP2Ac) contains a highly conserved C-terminal tail (^{304}TPDYFL^{309}) which is located at an important interface between the structural A and regulatory B-type subunit binding sites [9]. The PP2Ac tail can be modified by phosphorylation on the Y307 residue to alter the activity of PP2A and also methylated on the terminal L309 [14]. The importance of this carboxy-terminal methylation will be discussed in depth on page 7. The B-type subunit of PP2A serves as the regulator of PP2A function and consists of four unrelated families, namely B (B55/PR55), B' (B56/PR61), B" (PR72), and B"' (striatin family) [9, 15, 22, 23]. Each B-type family further encompasses multiple non-redundant isoforms and/or splice variants and, when assembled with the α or β isoforms of the A and C subunits, can form over 80 distinct PP2A holoenzymes to regulate a multitude of signaling pathways [24]. Additionally, some viral B-type subunits, including small DNA tumor viral proteins, middle tumor (MT) and small tumor (ST) antigens, can bind to PP2A at a region that overlaps the B-type subunit binding site and can alter the function of PP2A [25]. Many B-type family members are regulated both spatially and temporally within the cell; therefore, holoenzyme composition and function of PP2A are dictated by

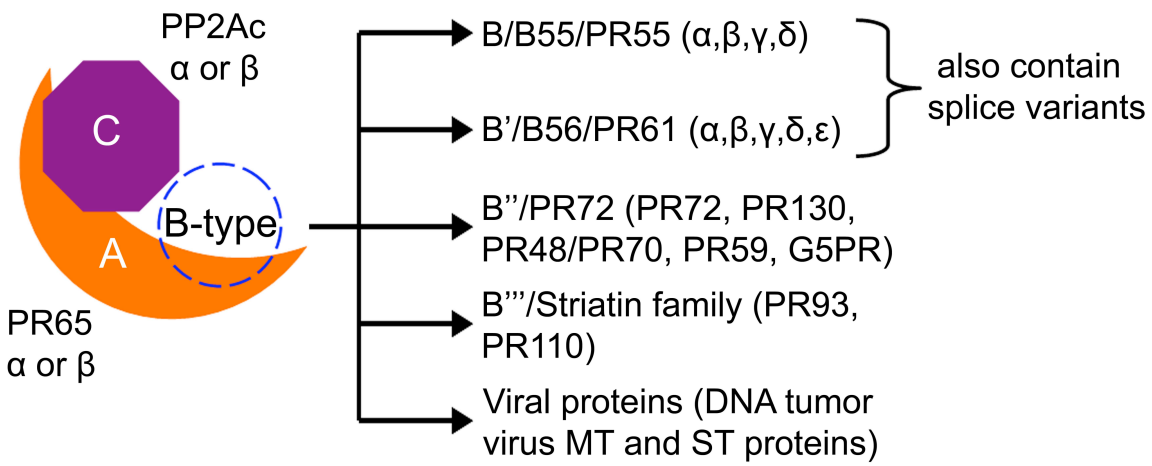


Figure 1. General schematic of PP2A holoenzyme composition. PP2A holoenzymes largely consist of a structural/scaffolding A/PR65 subunit, a catalytic C subunit (PP2Ac), and one of the various regulatory B-type subunit. Both the A and C subunits exist as α and β isoforms that are not completely redundant. The regulatory B-type subunit of PP2A, which provides enzyme specificity and thus controls PP2A function, consists of four unrelated families, namely B (B55/PR55), B' (B56/PR61), B'' (PR72), and B''' (striatin family). Furthermore, some B-type families contain multiple non-redundant isoforms and/or splice variants. In all, when each of the B-type subunits are combined with either the α and β isoforms of the A and C subunits, over 80 different combinations of PP2A holoenzymes can be assembled. In addition to the conventional B-type subunits, some DNA tumor viral proteins, middle tumor (MT) and small T (ST) antigens, can bind at a region on the PP2A A subunit that overlaps the B-type binding site. These oncoproteins act as viral B-type subunits and can alter the function of PP2A.

its location and by the availability of specific B-type subunits for PP2A assembly [26]. This mechanism of regulation allows PP2A to participate in a large variety of cellular processes, including transcription, translation, DNA replication, development, cell cycle progression and apoptosis [22], by distinctly opposing the action of a wide array of kinases.

Reversible Methylation of PP2A C Subunit

In addition to the large number of PP2A heterotrimer combinations provided by the B subunit isoforms to distinctly regulate a wide array of cellular pathways, PP2Ac can be covalently modified adding yet another mechanism of regulation [22]. As mentioned above, the carboxy-terminus of the PP2A C subunit (PP2Ac) can be reversibly methylated on its carboxy-terminal L309 residue [27-30], and this methylation serves as the most selective mechanism known to regulate PP2A complex formation [31]. Methylation and demethylation of PP2Ac is catalyzed by **L**eucine **C**arboxyl **M**ethyl**t**ransferase-**1** (LCMT-1) [32, 33] and **P**rotein Phosphatase **M**ethylesterase-**1** (PME-1) [34-36], respectively (Figure 2). The two enzymes have different subcellular locations with LCMT-1 more abundantly localized in the cytosol and PME-1 most abundant in the nucleus; therefore, the methylation levels of PP2Ac is spatially regulated [37]. S-adenosylmethionine-dependent (AdoMet) LCMT-1, first identified by isolation from bovine brain [28], is a highly conserved 38kDa enzyme that appears to be specific for methylating PP2A [38], although other closely-related PSTP family members, including PP4 and PP6, cannot be eliminated as potential LCMT-1 targets. Crystal structures of LCMT-1 associated with AdoMet and bound with PP2A reveal that the

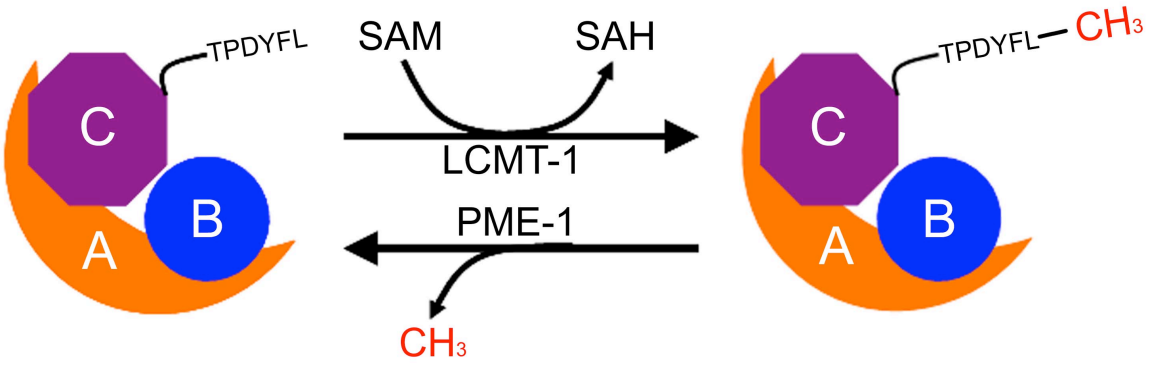


Figure 2. General schematic of reversible methylation of PP2Ac. Leucine Carboxyl Methyltransferase-1 (LCMT-1) and Protein Phosphatase Methylesterase-1 (PME-1) catalyze the methylation and demethylation of PP2Ac, respectively. The highly conserved carboxy-terminal tail of PP2Ac is methylated on its terminal leucine residue by S-adenosylmethionine-(SAM)-dependent LCMT-1, which results in a homocysteine byproduct (SAH). The opposing enzyme of PP2Ac methylation, PME-1, is serine esterase which utilizes a catalytic-activated serine as its nucleophile. The methylation status of PP2Ac regulates the assembly of the B-type subunits and, therefore, modulates PP2A substrate specificity and function.

flexible, 6-residue C-terminal tail of PP2Ac can enter an active site cavity within LCMT-1 in close proximity to the methyl group donor on the AdoMet. Moreover, in order for PP2Ac to span the depth of LCMT-1's active site cavity to access the AdoMet group, a minimum 6 residues must be able to enter the cavity, thereby highlighting the specificity of LCMT-1 for PP2Ac [33, 39]. The opposing enzyme of PP2A methylation, PME-1, is a 44kDa serine esterase with a conserved motif found in lipases that utilize a catalytic-activated serine as their nucleophile [35, 40]. PME-1 binds directly to PP2Ac to catalyze L309 demethylation and can also remain bound to an inactive pool of PP2A, which cannot be reactivated by LCMT-1 alone but requires PTPA, a PP2A peptidyl-prolyl cis/trans isomerase, to dissociate PME-1 from the inactive PP2A complexes [39, 41]. While the physical mechanism of PP2A methylation has been well-studied, the importance of this modification on PP2A regulation and function has only begun to be elucidated.

Role of PP2Ac Methylation

PP2Ac methylation is important for regulating the function and specificity of PP2A by differentially affecting the recruitment of the regulatory B-type subunits to the PP2A A/C dimer rather than affecting the PP2A catalytic activity directly [42-46]. In *Saccharomyces cerevisiae*, the importance of PP2Ac methylation for B subunit recruitment is particularly emphasized in studies utilizing PP2Ac methylation deficient mutants or deletions of the LCMT-1 homologue, Ppm1p. Since budding yeast contain only one B subunit homologue, Cdc55p, and one B' subunit homologue, Rts1p, studies using this simple model have exposed the critical nature of PP2Ac methylation for B

subunit binding. Reducing PP2Ac methylation in yeast by mutating the methylation residue or deleting the yeast methyltransferase homolog, Ppm1p, causes a dramatic loss of Cdc55p recruitment to the PP2A A/C dimer, and furthermore, greatly diminishes Rts1p binding [43, 45, 47, 48]. PP2Ac methylation also correlates with an enhanced binding of Cdc55p and Rts1p to the PP2A A/C dimer resulting in a 20-fold and close to a 2-fold increase, respectively, in heterotrimer formation with the two subunits [48]. Interestingly, reducing PP2Ac methylation correlated with an increased sensitivity to nocodozole, a microtubule destabilizing drug, thereby implicating PP2A methylation in the regulation of mitosis [43, 47]. This result was not surprising considering the fact that PP2Ac methylation fluctuates in a cell cycle-dependent manner [49, 50], and thus highlights the temporal regulation of this PP2Ac modification. In the mammalian system, similar results have been revealed for the impact of PP2Ac methylation on B-type subunit binding. Multiple studies have shown that PP2Ac methylation is important for the recruitment of some B and B' subunit members and is, in particular, critical for Bα subunit-containing heterotrimers [42, 44, 46, 51, 52]. Conversely, binding of the B"' members, SG2NA and Striatin, to the PP2A A/C dimer does not require PP2Ac methylation. In fact, these B-type subunits can bind regardless of the methylation status of PP2Ac [44] thereby making them "methylation-insensitive". Interestingly, the viral B type subunit, polyomavirus middle tumor (PyMT) antigen, can also bind to either the methylated or unmethylated form of PP2Ac [42, 44], and thus binds to PP2A A/C dimer in a methylation-insensitive manner. The importance of this finding will be discussed below in the DNA tumor virus section.

Mammalian studies involving the manipulation of PP2Ac methylation levels by altering LCMT-1 have exposed significant insights into the importance of methylation for regulating essential cellular processes. Knockdown of LCMT-1 through RNA interference (RNAi) in HeLa cells have revealed that PP2Ac methylation is critical for Bα subunit stability [53]. Furthermore, LCMT-1 knockdown prompts the Bα subunit to sequester the remaining pool of methylated PP2Ac at the expense of other subunits whose binding does not rely as critically on methylation [52]. Upon excessive LCMT-1 knockdown, PP2Ac methylation levels fall to a threshold that Bα subunit binding (and possibly other methylation-dependent subunits) cannot recover from, resulting in DNA condensation and fragmentation, cellular "blebbing", and apoptosis. Knockdown of LCMT-1 also rendered cells sensitive to nocodozole, which revealed significant mitotic and survival issues as seen by a large sub-G1 populations and cell division defects [52, 53]. In agreement with these findings, an LCMT-1 dominant negative mutant (LCMT-1 T29V) also attenuates cell proliferation in C6 glioma cells [54]. Interestingly, gene-trap knockout of LCMT-1 in mice results in embryonic lethality indicating that PP2Ac methylation is critical for mammalian development [53]. Lastly, recent findings have connected the down-regulation of PP2Ac methylation and Bα subunit-containing PP2A complexes to hyperphosphorylation of the Tau protein, a hallmark of Alzheimer's Disease [55-57]. Altogether, these findings emphasize the essential nature of PP2Ac methylation in regulating the normal function and assembly of the PP2A holoenzyme and provide an important foundation for further investigation of PP2Ac methylation in diseases, including cancer and Alzheimer's disease.

PP2A Function: A Pleotrophic Protein

With the large number of PP2A heterotrimeric combinations possible, it is not surprising that PP2A regulates a large variety of cellular processes within eukaryotic cells (Figure 3). PP2A has been reported to regulate transcription, translation, cell cycle progression, DNA replication, development, apoptosis, and transformation among many other processes [22, 23, 25]. Moreover, PP2A regulates some signaling cascades, including the ERK/MAPK and PI3K/AKT pathways, at multiple levels and in some cases, depending on the PP2A holoenzyme composition, can do so in both a positive and negative manner to elicit the proper cellular response [19, 23, 25, 58]. Over the past few decades, researchers within the field have sought to reveal the wide array of cellular processes regulated by PP2A and to identify the specific holoenzymes (regulated by B-type subunit specificity) that control each particular pathway. With the use of cell-permeable phosphatase inhibitors, viral B-type subunit proteins that inhibit PP2A, and later the development of RNAi to specifically knock down PP2A subunits individually, considerable strides have been made in matching specific PP2A complexes to particular pathways and processes. Although researchers are making significant progress in decrypting the pleotrophic nature of PP2A, there is still much to be determined about the complexity and function of the over 80 different enzyme combinations. Considering that PP2A is closely involved in regulating cellular decisions dictating growth and survival, there is a great potential of targeting specific PP2A complexes for cancer therapies.

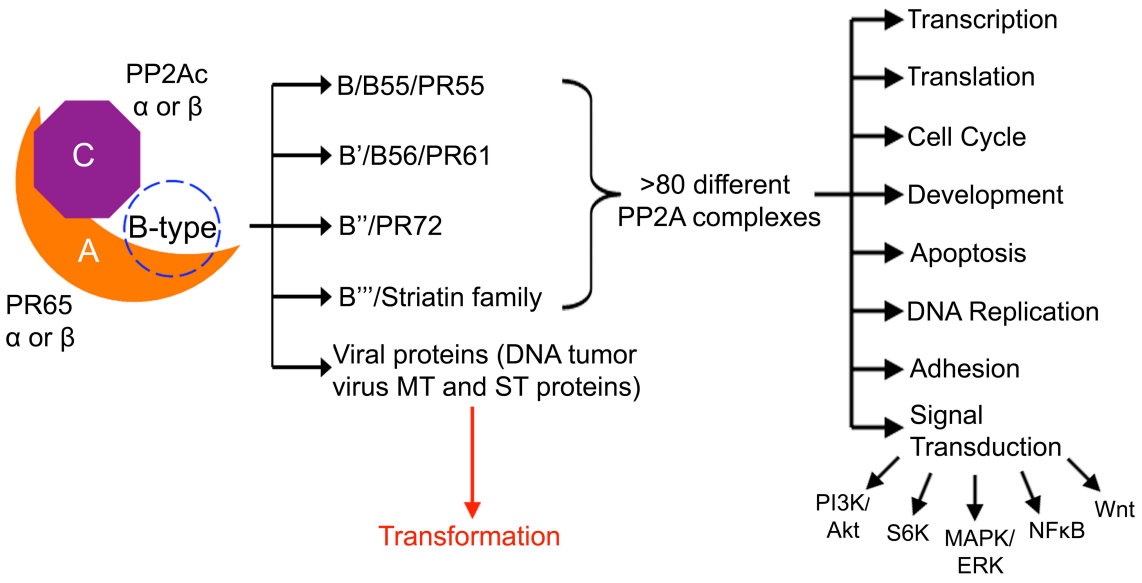


Figure 3. General schematic of PP2A functions. When each of the B-type subunits are combined with either the α and β isoforms of the A and C subunits, over 80 different combinations of PP2A holoenzymes can be assembled. This allows PP2A to distinctly oppose the actions of a wide variety of kinases and to participate in a multitude of cellular processes, including transcription, translation, DNA replication, development, cell cycle progression. Furthermore, selection and assembly of B-type subunits into PP2A complexes can direct the phosphatase activity of PP2A to positively or negatively regulate growth and survival pathways, including the Akt and ERK pathways, depending on PP2A holoenzyme composition. Viral proteins also act as B-type subunits and alter PP2A function, resulting generally in cellular transformation.

Akt/PKB Pathway

Akt/PKB, a versatile serine/threonine protein kinase downstream of PI3 kinase (PI3K), serves as a central regulator of signal transduction and dysregulation of this critical regulatory protein can lead to multiple cellular diseases including diabetes and cancer. Akt directs not only cellular growth and survival, but also nutrient metabolism and can regulate transcription factors; therefore, precise regulation of this protein is crucial for normal cellular function [59, 60]. Activation of Akt is carried out in three distinct steps: recruitment to the plasma membrane, conformational change, and phosphorylation of two activating residues [25]. Phosphorylation of Akt occurs on T308 in the activation loop and S473 in the C-terminal regulatory domain which leads to activation. While phosphorylation of both residues lead to full activation [61, 62], T308 phosphorylation is sufficient to partially activate Akt, while S473 alone is not. Recent studies have shown that PDK1 is the kinase for the T308 residue while PDK2, ILK, and possibly auto-phosphorylation are responsible for S473 phosphorylation [59, 63].

Conversely, dephosphorylation of these activating residues is important to negate Akt's function to prevent aberrant growth and survival; however, less is understood regarding this mechanism. Some studies have determined that PHLPP can dephosphorylate S473 while PP2A can dephosphorylate both the T308 and S473 residues to negatively regulate Akt [25, 64]. Regarding the role of PP2A in regulating the PI3K/Akt pathways, several studies have shown that inhibiting PP2A function via viral expression, siRNA knockdown, or the PP2A inhibitor okadaic acid, results in elevated Akt phosphorylation and activity [62, 65-69]. Furthermore, enhancing PP2A activity by ceramide treatment blocks Akt phosphorylation and activity [70-72]. Recently,

researchers have begun to elucidate the distinct PP2A holoenzymes, dictated by their specific B-type subunit association, that are responsible for Akt dephosphorylation. Some studies in mammalian systems have identified the B'/B56-containing PP2A complexes as a specific Akt phosphatase which can dephosphorylate both the T308 and S473 residues [69, 73]. Another study found that specifically knocking down B'β in murine adipocytes resulted in enhanced Akt T308 phosphorylation [74]. Additionally, others have shown that Bα-containing PP2A holoenzymes can dephosphorylate Akt on T308 [61, 75]. While these data provide initial key insights into understanding the relationship between PP2A and Akt function, there is still much to be determined, as it is likely that other B-type subunits, and possibly the modifications on PP2Ac, can direct PP2A to Akt to distinctly regulate critical downstream growth and survival pathways. Furthermore, considering that Akt is upregulated in multiple cancers [76], it is essential to identify how PP2A regulates this important protein.

As mentioned previously, Akt activation can direct a large number of growth and survival pathways through phosphorylation of downstream targets. One protein in particular, glycogen synthase kinase-3β (GSK3β), is a direct substrate of Akt that is implicated in the control of cell survival. GSK3β, a ubiquitously expressed Ser/Thr kinase in mammalian cells, can be phosphorylated on its Ser9 residue by activated Akt [77, 78]. Phosphorylation on Ser9 inhibits GSK3β activity toward more than 40 substrates including β-catenin, a key signaling protein in the canonical Wnt signaling pathway [77]. Therefore, when Wnt-activated Akt phosphorylates and inhibits GSK3β, β-catenin is not targeted for degradation by GSK3β phosphorylation and instead translocates to the nucleus to activate growth and survival transcription factors [79].

Hence, GSK3β activity is necessary to block prosurvival signals and induce apoptosis/anoikis and is thus considered a key tumor suppressor [77]. Interestingly, PP2A modulates the Wnt pathway in both positive and negative manners at various points. For example, B'-containing PP2A complexes can dephosphorylate and thus activate GSK3β thereby preventing Wnt-mediated transcription of prosurvival, progrowth genes [15, 23, 80]. Additionally, it has been demonstrated that PP2A-B'α and –B'γ complexes can reduce β-catenin levels and inhibit Wnt signaling [81-83]. Conversely, Bα-containing PP2A holoenzymes were found to directly interact with and dephosphorylate β-catenin to activate Wnt-mediated transcription [84]. While the balance of PP2A's dual role in regulating Wnt signaling is still being unraveled, the net effect of inhibiting PP2A via okadaic acid results in the stabilization of β-catenin, thus indicating that PP2A largely functions as a negative regulator of the Wnt signaling pathway [82]. Therefore, it is necessary to further explore how PP2A B subunit composition modulates this prosurvival pathway.

Akt-mTOR-S6 Kinase Pathway

As mentioned above, Akt is a highly pleotrophic protein that regulates a multitude of signaling cascades and cellular functions. The PI3K-Akt-mTOR pathway, in particular, is a potent conduit for regulating cell growth, proliferation, and survival and when dysregulated can contribute to the development of cancer [85]. mTOR (mammalian target of rapamycin), an evolutionarily conserved protein, is activated by various growth factors, cytokines, and nutrients to increase cell growth and proliferation by upregulating mRNA biogenesis, translation of ribosomal machinery, and protein synthesis [85, 86].

Two key downstream effectors of this process, 4E-BP1 and S6 kinase 1 (S6K1), are direct phosphorylation targets of mTOR and are commonly used as *in vivo* readouts for mTOR activity [85, 86]. Phosphorylation of the translational repressor protein, 4E-BP1, is inhibitory and promotes the release of 4E-BP1 from translation initiation factors resulting in activation of translational machinery. S6K1, on the other hand, is activated by mTOR-mediated phosphorylation and positively modulates cell growth and survival [85]. S6K1 encodes three proteins, p70, p85 and p31, through alternative translational start sites with p85 containing an NLS and considered the major S6 kinase protein in mammalian cells [87]. S6K1 is highly versatile in its functions and has been shown to coordinate transcription, translation, cell growth and size, and differentiation [88]. Ribosomal protein S6 (rpS6), a major target of S6K1, is a component of the 40S ribosomal subunit that is activated by S6K1-mediated phosphorylation and is a key player in directing cell growth and size through enhancing translation [85, 88, 89]. Interestingly, S6K1 is frequently overexpressed in certain cancers, including breast (via gene amplification [87]), and is implicated in brain tumor pathogenesis. Therefore, S6K1 meets the criteria as an attractive target for cancer therapies. However, no commercially available S6K1 inhibitors have been developed [89].

As established above, PP2A is a bona fide phosphatase for Akt and thus it can regulate the PI3K-Akt-mTOR pathway to downregulate the progrowth, prosurvival signals mediated by mTOR signaling. mTOR, however, can circumvent PP2A's negative regulation by phosphorylating PP2Ac on an inhibitory site to potentiate Akt-mediated activation of mTOR, thereby generating a positive feedback mechanism for the signaling cascade [88, 90]. Interestingly, studies using rapamycin, an inhibitor of mTOR, reveal

that S6K1 becomes dephosphorylated on residues not directly targeted by mTOR. This result is consistent with the idea that S6K1 can also be dephosphorylated by the actions of a phosphatase rather than solely through inhibition of the mTOR kinase [85]. These studies have also shown that rapamycin treatment and nutrient deprivation results in activation of PP2A, further supporting the possibility of PP2A as a critical phosphatase in the PI3K-Akt-mTOR pathway [88, 91]. Additionally, inhibiting PP2A (and PP1) by calyculin A treatment of cells corresponds to significant increases in 4E-BP1 and S6K phosphorylation [91]. Recent investigations have provided strong evidence that PP2A is, indeed, a phosphatase for S6K1 and can stably associate with S6K1 to dephosphorylate and thus inactivate the progrowth and prosurvival effects of this kinase [25, 87, 92]. To further delineate which PP2A complexes provide the substrate specificity for S6K1, one group developed PP2A-B' knockout Drosophila. The knockout flies had elevated S6K1 phosphorylation levels, reduced response to nutrient changes, and reduced life spans [93]. Additionally, the same group showed that S6K1 is a direct substrate of human PP2A-B' and determined that B'γ-containing PP2A complexes specifically promoted p70 S6K1 dephosphorylation in the mammalian system. Other mammalian studies have demonstrated that Bα-containing PP2A complexes bind and dephosphorylate p70 S6K1 [94]. While these studies have provided some key glimpses into PP2A-mTOR pathway connections, further supporting data is needed for understanding how modulating PP2A can provide an additional foundation for anti-cancer therapies.

ERK/MAPK Pathway

The ERK/MAPK pathway is a modular pathway that regulates a wide array of cellular processes including cell growth, proliferation, apoptosis/survival, differentiation and transcription. The pathway is typically stimulated by extracellular signals (mitogens and growth factors) at the plasma membrane which sequentially activate the Ras-Raf-MEK-ERK kinase signaling cascade [25, 95]. Aberrant activation of the ERK/MAPK cascade is oncogenic and is a required component for the transformation of human cell lines [95]. Furthermore, mutations that constitutively activate the ERK/MAPK pathway, particularly in Ras and Raf, are found in a wide variety of cancers including lung, colon, pancreatic, thyroid and melanoma cancers [96]. While the ERK/MAPK pathway is regulated by multiple phosphatases, PP2A appears to be one of the most influential players, as it can not only stably bind to Raf and ERK to regulate the pathway at multiple points within the cascade, but can also regulate the pathway in both positive and negative manners [69, 97]. When PP2A is inhibited through viral oncoprotein expression, okadaic acid treatment, or siRNA knockdown, studies have shown that MEK and ERK are hyperphosphorylated and thus activated independently of extracellular signals [98, 99]. Researchers have attempted to elucidate which B-type subunits direct PP2A's regulation of the ERK/MAPK pathway; however, our current understanding is still in its early stages. In Drosophila and mammalian systems, B'-containing PP2A complexes can dephosphorylate ERK and thus inhibit its activity [73, 100, 101]. Interestingly, some studies have found that the Bα and Bδ-containing PP2A complexes negatively regulate ERK phosphorylation [69], while others have determined that the same holoenzymes positively regulate the ERK/MAPK pathway at the level of Raf [97]. These findings

accentuate the complexity of this system and emphasize that both positive and negative feedback mechanisms between PP2A and ERK/MAPK are utilized in this signaling cascade to regulate cellular proliferation and survival responses.

Cell Cycle

The cell cycle, a process which directs the creation of two genetically identical daughter cells, is orchestrated by a series of well-regulated events that are highly conserved from yeast to humans and are commonly misregulated in many cancer lines and tumors [102-106]. Reversible protein phosphorylation is considered one the most critical driving forces for cell cycle progression and through subtle changes in the balance of kinases and phosphatases, dynamic alterations in the cell can be both temporally and spatially regulated to ensure the appropriate succession of events [107, 108]. While research has substantiated the importance of activating kinases as a positive driving force for cell cycle progression, only more recently has the relevance of protein phosphatases for cell cycle processes become evident [102]. Current findings have revealed that phosphatases, like their counterbalancing kinases, significantly influence the regulation of growth, DNA replication, damage checkpoints, and mitotic progression [102, 107, 109]. PP2A is one of the major protein phosphatases involved in regulating the cell cycle at both interphase as well as mitosis. During interphase, PP2A can direct the G0/G1 transition, G1/S phase transition, and the DNA damage checkpoint depending on the PP2A holoenzyme composition [109-111], and while there is accumulating evidence suggesting additional roles for PP2A during interphase, the remaining discussion in this

section will be briefly focused on PP2A's involvement in mitotic events including mitotic initiation (G2/M transition), prometaphase/anaphase transition, and mitotic exit.

From yeast to mammalian cells, entry into mitosis is a highly conserved, well-regulated event that is triggered by the activation of the mitosis-driving Cdk1/cyclin B complex or MPF (mitosis promoting factor). MPF activation results in the increased phosphorylation of a multitude of mitotic substrates which direct various cellular restructuring events including cell rounding, nuclear envelope breakdown, chromosome condensation, and assembly of mitotic spindles [103, 104, 108, 112-114]. PP2A was first implicated in cell cycle regulation by the finding that inhibition of PP2A caused premature mitotic entry. Concomitantly, upon PP2A inhibition, an increase in Cdk1 activity occurs resulting in increased MPF activation, thereby linking PP2A as a major negative regulator of mitotic initiation [102, 104]. Recent studies have determined that PP2A is involved in mitotic entry at multiple levels and in some cases have revealed which distinct PP2A holoenzymes direct specific events in regulating the cell cycle. PP2A maintains cells in interphase by downregulating Cdc25, an activator of Cdk1, while also upregulating Wee1, an inhibitor of Cdk1, thereby indirectly inhibiting MPF activation [104]. Specifically, PP2A-Bδ complexes were shown to direct these regulatory events and when added to or depleted from Xenopus extracts, caused delayed or advanced entry into mitosis, respectively. In addition to Bδ, B'δ inhibits Cdc25 by enhancing 14-3-3 affinity for Cdc25 thereby blocking its localization to the nucleus. Once the DNA damage checkpoint is satisfied, PP2A-B'δ dissociates from Cdc25 resulting in 14-3-3 dissociation and Cdc25 localization to the nucleus where it can activate Cdk1/Cyclin B complexes [105, 107, 113]. These results indicate that specific

forms of PP2A complexes direct mitotic entry, and that only when distinct cellular requirements are met does PP2A become downregulated to release the cells into mitosis, further supporting the role of PP2A as a critical tumor suppressor.

Once cells commence mitosis, DNA condenses and chromosomes are "captured" by opposing mitotic spindles in order to separate the sister chromatids into separate daughter cells. The bipolar attachment of the spindle microtubules to the chromosomal centromeres represents a critical checkpoint for mitosis and is required for the subsequent sister chromatid separation during the metaphase to anaphase transition. Before anaphase, when microtubules have yet to completely attach to the chromosome kinetochore (also known as the spindle checkpoint), cohesin, a multisubunit protein complex, mediates sister chromatid cohesion to assure that symmetrical segregation of DNA will occur. After appropriate attachment of all mitotic spindles and chromosomal alignment, cohesin is phosphorylated resulting in cleavage to allow separation of the chromatids (process reviewed in [115]) . Recently, it was determined that PP2A is a negative regulator of chromatid separation at two distinct points. First, PP2A-B' complexes directly protect premature cohesin cleavage by being recruited to the centromere by Shugoshin 1 (Sgo1) to oppose the phosphorylation of cohesin by Plk1 and Aurora B. This allows the centromeres to maintain their pool of persistent cohesin, thereby stabilizing sister chromatid adhesion and preventing faulty chromosomal separation [107]. Subsequent research determined that the PP2A-B'α holoenzymes were specifically involved in this protective mechanism [116] although other B' family members cannot be ruled out. PP2A can also protect against premature cohesin cleavage indirectly at the level of securin. Securin negatively regulates sister chromatid separation by binding to and

inhibiting the cohesin protease, Separase. PP2A-Bδ complexes have been shown to stabilize securin thus preventing premature cohesin cleavage by separase [117]. Lastly, some data has suggested that PP2A-Bα complexes can negatively regulate the spindle checkpoint, and that PP2A methylation (via LCMT-1) is also a critical factor influencing chromatid separation [53]. The finding that LCMT-1 is a required enzyme for this process indicates that "methylation-sensitive" PP2A holoenzymes, that is, complexes that rely on PP2Ac methylation to form, are important regulatory complexes in this process. Moreover, this finding is interesting considering that PP2Ac methylation fluctuates throughout the cell cycle [49] and that nearly all of the B-type subunits found to be involved in most of the above-mentioned mitotic processes are, to some degree, methylation-sensitive.

Once cells undergo proper chromosome alignment at the metaphase plate, the cell must be biochemically reset to commence cytokinesis and to reenter interphase. Exiting mitosis involves the activation of separase to cleave the sister chromatids, inactivation of the MPF, the reversal of a multitude of mitotic phosphorylations, and the reorganization of cellular structures such as the Golgi apparatus and endoplasmic reticulum to reduce spatial interference with the mitotic spindle and separating chromosomes. Briefly, once chromosomes properly align, Cdk1 activates the APC (anaphase promoting complex) which causes the destruction of 1) Securin, thus releasing separase to cleave the centromeric cohesin, and 2) Cyclin B, thereby reducing the Cdk1/Cyclin B (process reviewed in [108]). This decrease in MPF presumably reduces the inhibitory effect on PP2A, specifically Bδ- and B'δ-containing complexes, which releases the phosphatase to enhance Wee1 and reduce Cdc25 to further counteract MPF. Furthermore, other data has

revealed that the B55-containing PP2A complexes also counteract Cdk1 by recognizing the Cdk1 substrate consensus sequence to remove mitotic phosphorylations that control cellular reorganization upon mitotic exit [118]. Specifically, Bα subunit confers the specificity towards Cdk1 substrates in human [119], while Bδ does so in Xenopus [120], and depletion of the subunits in the respective system causes mitotic delay and eventual cell death with failure to progress through mitotic exit. Although normal cells will typically undergo apoptosis if they fail to properly traverse through mitosis, cancer cells, in contrast, are defined by their ability to circumvent this barrier. Therefore, modulation of PP2A may offer new insights for developing novel anti-cancer therapies.

Apoptosis

Apoptosis, the process of genetically programmed cell death, plays a key role during cell turnover in embryonic development and tissue homeostasis and is a critical mechanism in expediting the elimination of abnormal or harmful cells. Dysfunction of apoptosis is found to be prevalent in multiple disease systems including cancer, autoimmune disorders, and neurodegenerative diseases [121, 122]. Apoptosis is initiated either through extracellular death ligand-receptor signaling (extrinsic) or intracellular targeting of the Bcl-2 family of signal transducers (intrinsic), and is associated with precisely orchestrated morphological changes including cell blebbing and shrinkage, chromatin condensation, and DNA fragmentation [58, 122]. Initiation and progression of apoptosis is coordinated, like many cellular processes, by reversible phosphorylation of apoptosis-mediating proteins and thus depends on the activity of kinases and phosphatases [123]. Considering PP2A's extensive involvement in various survival

pathways mentioned previously, it is not surprising that it is also an essential player in the regulation of apoptosis. While PP2A has been linked to both the positive and negative regulation of apoptosis, depending on the cell system and conditions [124], the majority of current evidence emphasizes its role in promoting apoptosis, thereby supporting its well-accepted function as a tumor suppressor. Therefore, for the purposes here, the role of PP2A as a pro-apoptotic protein will be briefly reviewed to provide evidence to support its tumor preventative role.

Transduction of apoptotic signaling is primarily mediated by the Bcl-2 family of regulatory proteins, which consists of both pro-apoptotic members including, but not limited to, Bax, Bad, and Bim and anti-apoptotic members, which include Bcl-2 and Bcl-XL [121, 123, 125]. The Bcl-2 members mediate apoptotic signaling by their ability to regulate mitochondrial release of mitochondrial cytochrome c (cyt c), which when released can activate caspase proteins to cleave substrates to execute apoptotic events related to cell disassembly and destruction [121, 126]. The regulation and function of Bcl-2 family members is largely dictated by their phosphorylation status and their homo- and heterodimerization, which can either trigger or block apoptosis depending on extracellular or internal signals [58, 121, 127, 128]. For example, during survival signaling, the anti-apoptotic family members, Bcl-2 and Bcl-XL are hyperphosphorylated and function as homodimers to block mitochondrial cyt c release resulting in cell survival [121, 123, 129]. Furthermore, Bcl-2 can heterodimerize with and inactivate the pro-apoptotic family member Bax to block its pro-apoptotic activity [128, 130]. In contrast, heterodimerization of Bcl-2 or Bcl-XL with the pro-apoptotic family member Bad, results in Bad-mediated inactivation of Bcl-2 and Bcl-XL, thereby blocking their anti-apoptotic

function to promote cell death [122, 123]. In most cases, dimerization of Bcl-2 family members is regulated by their reversible phosphorylation and can result in either a pro- or anti-apoptotic response depending on which members are modified. Dephosphorylation of these family members appears to play a critical role for initiating apoptosis and several serine/threonine protein phosphatases including PP1 and PP2A have been confirmed as major phosphatases in this process [121-123].

PP2A has been implicated in dephosphorylating a number of Bcl-2 family members to promote apoptosis [121-123] thereby protecting cells against harmful or aberrant growth as observed in cancer. First, PP2A-B and B'-containing complexes can associate with and dephosphorylate the anti-apoptotic Bcl-2 protein at the mitochondrial membrane, resulting in inactivation of Bcl-2 and initiation of apoptosis [131-134]. Second, when pro-apoptotic Bad is phosphorylated by Akt or S6K, it is bound to 14-3-3 in the cytoplasm and cannot translocate to the mitochondria to dimerize with and inhibit Bcl-2/Bcl-XL's anti-apoptotic function [123, 135]. PP2A can counteract this phosphorylation on Bad resulting in its translocation to, and subsequent association with, Bcl-2 or Bcl-XL resulting in the inactivation of the anti-apoptotic proteins [122, 135, 136]. Third, when pro-apoptotic Bax is phosphorylated, Bcl-2 associates with it to inactivate its pro-apoptotic function. Reversal of this phosphorylation on Bax by PP2A disrupts the Bax/Bcl-2 heterodimer which then liberates Bax to initiate apoptosis [130]. In each case presented here, PP2A serves as an upstream positive regulator for apoptotic signaling; however, PP2A is also implicated downstream as both a substrate and a regulator of effector caspases [122]. Caspases are cysteine proteases that are activated by irreversible proteolytic cleavage and mediate the morphological changes mentioned

above that are associated with commitment to apoptosis [123]. Caspase 3, in particular, is considered one of the most downstream apoptosis signaling proteins and has been extensively studied. Recently, PP2A A subunit was found to be a caspase-3 substrate and when cleaved caused an increase in PP2A activity which correlated with cell commitment to apoptosis [137]. In turn, PP2A dephosphorylates and increases caspase-3 activity thus highlighting a positive feedback loop for the initiation of apoptosis [138].

Recent studies have linked PP2A to mediating upstream regulators of apoptosis, including the FOXO proteins and p53. The FOXO proteins function as transcription factors to upregulate pro-apoptotic proteins including some of the Bcl-2 family members [139]. Specifically, FOXO1 and FOXO3a are Akt substrates that when phosphorylated cannot translocate to the nucleus to upregulate pro-apoptotic proteins. Furthermore, okadaic acid treatment prevents FOXO1 and FOXO3a dephosphorylation and subsequent apoptosis, implicating PP2A as the likely pro-apoptotic activator of these FOXO proteins. Recent studies have shown that PP2A can directly interact with and dephosphorylate both FOXO1 and FOXO3a on Akt phospho-sites resulting in nuclear translocation and initiation of apoptosis [139, 140]. Additionally, the proapoptotic protein, Bim, is a target of both FOXO1 and FOXO3a and is inhibited when PP2A activity is blocked, correlating with reduced death. Therefore, these studies indicate that PP2A is essential in upregulating pro-apoptotic genes via FOXO activation [139].

With regards to p53-dependent apoptosis, PP2A has been implicated in enhancing p53 stability [141-143], which enhances expression of proapoptotic Bcl-2 family members including Bax and Bid as well as Apaf-1, a co-activator of caspase 9 [144-146]. Specifically, PP2A-B'γ-containing complexes dephosphorylate and stabilize p53 in

response to DNA damage [142]. Additionally, PP2A complexes containing B'ε can trigger p53-dependent apoptosis and can activate caspase 3 to promote substrate cleavage [147]. Furthermore, as mentioned previously, activation of PP2A also inhibits Bcl-2 phosphorylation which enhances p53/Bcl-2 interactions resulting in increased apoptosis [148]. Overall, while a complete perspective of PP2A's role in apoptosis is still being constructed, current insights presented here highlight the positive role of PP2A in apoptosis and provide support for a tumor suppressive role for PP2A. Together, these findings emphasize the specialized nature of the PP2A B-type subunit in directing PP2A function and substantiate the idea that modulation of PP2A may provide valuable insights for developing future therapeutic interventions for cancer.

Cancer and PP2A

In 2011, cancer ranked as the second most common cause of death in America with an expected one and a half million new cases and over a half million cancer-related deaths. While 5-year survival rates have significantly increased in recent years due to earlier detection and more effective treatments, cancer-related deaths are projected to surpass heart disease-related mortalities (the current #1 cause of death) shortly [149]. Cancer is defined as a disease by which cells can uncontrollably and indefinitely grow, replicate, and survive by evading the protective cellular mechanisms. These aberrant cells are characterized by several "hallmarks" that encompass the complexity of their transformative properties and include: evasion of cell death, self-sufficiency in growth signals, insensitivity to anti-growth signals, unlimited replication potential, sustained angiogenesis, and invasion and metastasis [150]. Furthermore, recent reviews have

proposed that genomic instability and the ability of cancer cells to evade immune surveillance should also be acknowledged as hallmarks of cancer [151, 152]. These characteristics not only encompass our understanding of the necessary steps required for a normal cell to transition into the malignant state but they also provide a general roadmap as to how to develop effective therapeutic strategies to overcome the extensive complexity of the disease. Considering that PP2A has been linked to many cell processes that prevent the cell's acquisition of several of these proposed hallmarks, it is not surprising that PP2A is considered a tumor suppressor.

PP2A: Evidence for a Tumor Suppressor

Some of the initial evidence that hinted to a possible tumor suppressive function for PP2A was based on the discovery that a marine-sponge toxin/compound, okadaic acid (OA), could bind to PP2Ac to potently block PP2A's enzymatic activity and promote tumorigenesis in mouse models [153-156]. This enhanced tumorigenesis correlated with increased expression of proto-oncogenes, c-fos and c-jun [157], as well as increased phosphorylation of a number of cellular proteins involved in cell cycle activation and progrowth, prosurvival signaling cascades including MAPK and PI3K/Akt pathways [22, 158, 159]. Okadaic acid, however, also inhibits several other phosphatases (PP1 and PP4), making the assignment of okadaic acid's effects to PP2A premature. Regardless, the extensive data acquired from studying the effects of OA has laid the foundation for our current understanding of PP2A's broad range of functions in the cell and has provided critical insights into its tumor suppressive capabilities. In support of the tumor promoting effect of OA, the discovery of endogenous cellular inhibitors of PP2A, SET

and CIP2A in particular, have also provided key evidence for PP2A's role as an inhibitor of transformation [24]. SET, first discovered as a fusion gene involved in myeloid leukemogenesis, is an oncoprotein which can regulate cell cycle, apoptosis, and gene transcription and does so, in part, by binding to and inhibiting PP2A function. The SET oncoprotein is found to be selectively and highly expressed in rapidly dividing cells and is overexpressed in multiple tumor types including Wilms' tumor, hepatoma, choriocarcinoma, and several types of leukemia (for review, see [160, 161]). Interestingly, overexpressing the PP2A C subunit or knocking down SET in some leukemia cells results in reduced cell proliferation, decreased activation of Akt, ERK, and c-Myc (known targets of PP2A), and inhibition of tumorigenesis [162, 163]. Similarly, expression of CIP2A also promotes transformation by potently inhibiting PP2A and Akt-associated PP2A activity and stabilizing proto-oncogenic c-Myc [24]. Recent studies have shown that knocking down CIP2A in HeLa cells can reduce proliferation and the formation of tumors in mice. Furthermore, overexpression of CIP2A in immortalized HEKTER cells confers on the cells the ability to grow and survive in anchorage-independent conditions, a general hallmark of transformation [17]. Not surprisingly, CIP2A is expressed less in normal tissues but is highly expressed in numerous tumor types including, head and neck, colon, lung, liver, breast, and gastric cancer [24, 164]. Therefore, it appears that inactivation of PP2A confers transformative phenotypes to a vast number of cell types and may represent a general key requirement for the initiation and progression of some types of cancer.

In addition to the common occurrence of PP2A endogenous inhibitor upregulation in numerous cancer lines, researchers have also found that some tumors have mutations

of the PP2A subunits that negatively affect holoenzyme formation, thus implicating specific subunits as tumor suppressors [9]. Sequencing of various human tumor samples identified mutations/alterations in the PP2A scaffolding Aα and Aβ subunits that included deletions, point and frameshift mutations, and splicing abnormalities [22]. Specifically, PP2A-Aβ mutations were reported in 15% of primary colon tumors, 15% of primary lung tumors, and 6% of lung tumor-derived cell lines [165]. Another study reported a reduction in PP2A-Aβ expression in 50% of 34 tumor lines tested [166]. Mutations in the Aα subunit gene have also been revealed in melanoma, breast, and lung carcinomas [167]. Additional studies confirmed that both the PP2A Aα and Aβ genes were genetically altered at low frequency in a number of primary human tumors and also determined that some A subunit mutants were deficient in binding to the catalytic C subunit as well as to the regulatory B' subunit [167-169], with B'γ being most affected [9]. Interestingly, truncations in the B'γ subunit correlated with a higher metastatic state in melanoma [170] and this subunit was also reported to be absent in 10 lung cancer lines thus highlighting its tumor suppressive capability [171]. Although mutations in the PP2A C subunit have never been reported in human cancer cells due to its indispensable requirement for cell survival [22], and little evidence exists for B subunit mutations, the fact that A subunit mutations that affect PP2A holoenzyme formation occur at a significant rate indicates that proper PP2A assembly is tumor suppressive. Therefore, disrupting PP2A holoenzyme assembly may be one oncogenic mechanism that occurs to promote transformation in some cell types. In fact, this hypothesis appears to be validated by small DNA tumor viruses which mediate transformation, in part, through the disruption of PP2A assembly.

DNA Tumor Viruses: Targeting PP2A Assembly

The study of small DNA tumor virus-mediated transformation has proven to be an invaluable tool for understanding the mechanisms by which cells transition from the normal state to an oncogenic phenotype and has provided key insights into the tumor suppressive role of PP2A (for review, see [172]). In fact, the study of polyomavirus and simian virus 40 (SV40) provided the first evidence that implicated PP2A as a tumor suppressor [173] and have revealed that inhibition of PP2A activity is an additional alteration required to drive immortalized cells towards a transformed state [17]. DNA tumor viruses promote transformation through the expression of their tumor antigens genes which encode dominant-acting oncoproteins that bind a variety of cellular targets to essentially hijack normal cellular processes including apoptosis, cell cycle, and signal transduction [14]. Incidentally, most cellular proteins that are targeted by the tumor antigens are involved in tumor suppressive functions and their deregulation has often been implicated in spontaneously arising tumors [23, 174]. A unifying feature of the aforementioned DNA tumor viruses is their ability to target PP2A to inhibit the phosphatase activity by binding directly to the PP2A A/C dimer and functioning as a non-canonical B-type subunit [172]. In many cell types, this inhibition of PP2A activity results in the deregulation of the cell cycle, and the upregulation of antiapoptotic proteins and multiple signaling pathways involved in cell growth and survival, ultimately leading to transformation. Of note, only a specific subset of PP2A complexes are targeted by the tumor antigens, and therefore, only the functions of those particular tumor antigen-targeted PP2A holoenzymes are disrupted [98, 171, 173, 175]. While the specifics of which PP2A complexes are being targeted is still under investigation, there is growing

evidence that the tumor antigens promote transformation by specifically targeting and replacing some members of the B and B' regulatory families [171].

Simian Virus 40 (SV40)

As stated above, the study of DNA tumor viruses has provided powerful insights into the functions of PP2A and into the molecular basis for transformation. SV40, in particular, has been one of the best studied members of the Polyomaviridae family (for review, see [172, 174]). SV40's 5kb genome consists of an early region (SV40 ER) which encodes three alternatively spliced proteins, the Large Tumor antigen (LT), the Small Tumor antigen (ST), and the 17kT antigen, and a late region which encodes the viral capsid proteins. Both regions can be expressed in "permissive" cells which allows for viral replication and assembly resulting in eventual cell lysis and viral spread. In "non-permissive" cells, only the early region, which contains the T antigen oncoproteins, is expressed resulting in transformation and subsequent tumor development. Therefore, the study of these cells has provided key insights into the process of transformation. The SV40 LT (SVLT) and ST (SVST) oncoproteins share identity in 82 amino acids in their N-terminal domains and deviate in the C-terminal sequences due to alternative splicing. SVLT contains multiple domains including a nuclear localization signal (NLS), DNA binding domain, LxCxE motif, and p53 binding domain which allow the oncoprotein to bind to and inhibit p53 and pRB activity. This alone was thought to be sufficient to transform rodent cell lines until mutational studies revealed SVST's requirement for full transformation. These studies found that deletion mutants in an SVST-specific exon

could no longer elicit transformed phenotypes and therefore it was concluded that both SVLT and SVST were required for robust transformation.

Early studies of SV40 revealed that the SVST antigen could bind specifically to the PP2A A subunit [173] at the amino-terminus (HEAT repeats 3-7) overlapping the B-type subunit binding site [176, 177]. This association was later found to correlate, in general, with the inhibition of PP2A activity and, in many cell lines, the stimulation of progrowth, prosurvival pathways resulting in cellular transformation [23]. Interestingly, mutational studies examining SVST function mapped out distinct N-terminal, PP2A–independent and C-terminal, PP2A-dependent domains, the latter region being essential for PP2A binding and for SVST-mediated transformation, thereby supporting the tumor suppressive role of PP2A [98, 178]. Moreover, although the PP2A-independent domain does not promote transformation and is not itself required for interaction of SVST and PP2A [178], it does enhance SVST's affinity for PP2A [179]. The current knowledge on the function of the PP2A-independent portion of SVST is quite limited; however, it has been linked to the transactivation of cyclin A involved in cell cycle progression [180] and also possesses chaperone activity from its J domain [181]. A much greater appreciation exists for SVST's role in targeting of PP2A and has expanded our understanding of how DNA tumor viruses overcome the negative role of PP2A to promote transformation.

Expression of SVST has been implicated in promoting transformation though the deregulation of PP2A-modulated processes including the cell cycle, cell proliferation, survival, and apoptosis, and also through supporting SVLT tumor promoting functions by blocking PP2A's negative regulation of SVLT [172]. Several studies have revealed that SVST expression stimulates both MEK and ERK activity resulting in enhanced MAPK

pathway activation and increased proliferation [98, 182]. SVST also promotes proliferation through upregulating proteins associated with cell cycle progression, including cyclin D1, cyclin A, and cyclin B [183, 184]. Other studies have implicated SVST in upregulating survival signaling and its expression has been reported to correlate with increased Akt phosphorylation and activation, stabilization of c-Myc, upregulation of anti-apoptotic targets, and suppression of pro-apoptotic FOXO transcriptional activity [172, 174, 185, 186]. Additional consequences of SVST expression are the upregulation of integrin signaling, microtubule destabilization, and F-actin rearrangements to alter cellular adhesion thus supporting anchorage-independent growth and survival [187]. Each of the SVST-induced alterations mentioned corresponds to a specific hallmark of cancer, and therefore, supports the role for PP2A as a tumor suppressor. However, because only a subset of the PP2A-mediated cellular processes and pathways are altered during SVST expression, and because complete inhibition of PP2A is lethal, SVST must target only a specific population of PP2A holoenzymes (for review, see [187]). Indeed, recent evidence has provided some support for this theory and has shown that SVST likely competes with several PP2A B and B' family members for the PP2A A subunit binding site. For instance, it is reported that expression of SVST reduces the PP2A Bα regulatory subunit and this correlates with increased MAPK activation [98, 188, 189]. Furthermore, based on the high sequence homology between Bα and Bδ [190], and the fact that SVST targets cell cycle machinery, a process heavily regulated by Bδ, this subunit may also be targeted by SVST. SVST expression also stabilizes c-Myc; therefore, it is presumed, although not confirmed, that B'α, a major c-myc phosphatase, is targeted by the SVST [191, 192]. Lastly, selective knockdown of the B'γ in immortalized HEK cells

(HEKTER) partially recapitulated the transformative effects of SVST and overexpression of B'γ reversed SVST-mediated transformation, indicating that this subunit it likely a target of SVST [171]. Altogether, these current findings suggest that SVST selectively inhibits a distinct subset of PP2A complexes; however, further research is necessary to fill the gaps in existing knowledge to better understand the nature of these targeted complexes and how their inhibition promotes transformation.

HEKTERASB56γ: A Genetically Defined Transformation Cell Line

In 2004, the Hahn and Pallas laboratories resolved to identify specific PP2A complexes targeted and inhibited by SVST during human cellular transformation [171]. In contrast to most rodent cells, which can be transformed by coexpression of SVLT and an oncogenic allele of H-Ras [193, 194], human cells are more difficult to transform and require additional components to become immortalized and tumorigenic [178]. In addition to SVLT, expression of hTERT, the human telomerase catalytic subunit, and H-Ras immortalizes human epithelial kidney cells resulting in HEKTER cells. While H-Ras is oncogenic, it does not significantly promote anchorage-independent growth or tumor formation in HEKTER cells. Expression of SVST in the HEKTER line, however, is sufficient to transform the cells and to promote tumor growth in immunodeficient mice. Therefore, HEKTER cells represented a well-defined experimental system for studying transformation in human cells [178] (Figure 4). In place of SVST, Hahn and colleagues used antisense RNA (AS) to target and suppress the expression of the PP2A Bα and B'γ subunits in an attempt to recapitulate SVST-mediated transformation [171]. Surprisingly, the group found that suppression of Bα, a known target of SVST, in HEKTER cells did

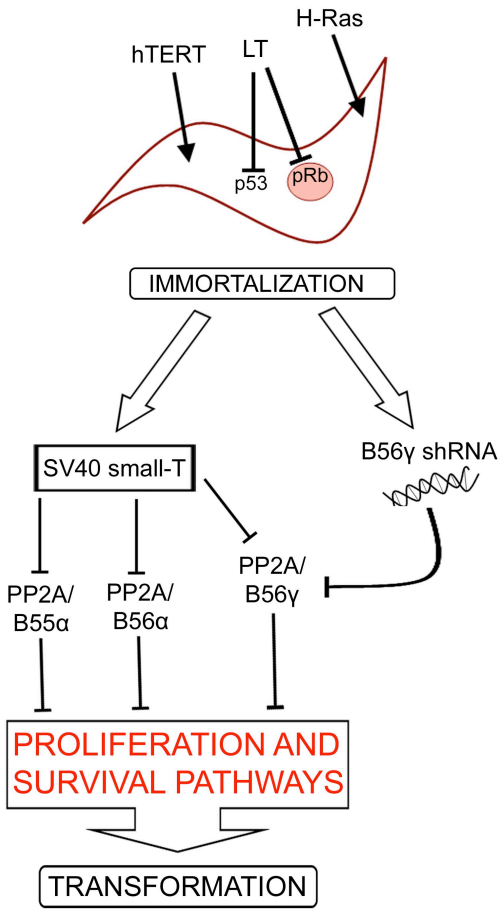


Figure 4. The HEKTER experimental cell model: Immortalization and transformation of human embryonic kidney (HEK) cells. Expression of 1) SV40 large T (LT), which inhibits antigrowth/antisurvival proteins p53 and pRb, 2) hTERT, the human telomerase catalytic subunit, and 3) oncogenic H-Ras, which activates the ERK/MAPK pathway, immortalize HEK cells resulting in HEKTER cells. Of note, while H-Ras is a transforming oncoprotein, it does not significantly promote anchorage-independent growth or tumor formation in HEKTER cells. Expression of SV40 small T (SVST) in HEKTER cells is sufficient to transform the cells by inhibiting multiple PP2A complexes involved in regulating proliferation and survival. Knockdown of the PP2A B'γ subunit, a subunit targeted by SVST, in HEKTER cells (HEKTERASB56γ), partially recapitulates SVST-mediated anchorage-independent growth and thus transformation.

not promote transformation and the resultant cells (HEKTERASB55α) could not form tumors in mice. Suppression of the B'γ subunit in HEKTER cells (HEKTERASB56γ), however, did confer the ability to grow in anchorage-independent conditions (soft agar) and also to form tumors in mice, albeit to a lesser extent than SVST-expressing HEKTER cells. These results support the hypothesis that SVST promotes transformation by targeting specific PP2A complexes and, considering the fact that targeting B'γ only partially recapitulated the SVST effects [171], also suggest that other B-type subunits are likely replaced by SVST to drive transformation. To this point, it remains to be determined if targeting multiple B-type subunits simultaneously will close the gap in the level of transformation between ST-expressing HEKTER cells and HEKTERASB56γ cells. It is possible that while SVST targets the PP2A Bα subunit, its contribution to transformation may be dependent on the concurrent suppression of the other SVST-targeted B-type subunits.

In another study by the Pallas group [184], a comparison between the ST-expressing HEKTER cells and HEKTERASB56γ identified similarities and differences in their gene expression profiles. First, a set of 99 genes was revealed to be similarly regulated by SVST expression and B'γ knockdown, including Akt and MAPK pathway activation, upregulation of antiapoptotic effectors, and the induction of cyclins, and likely represent some of the most essential changes for SVST-mediated transformation. The same study also determined that both lines shared a positive effect on integrin signaling which was confirmed to be essential for their anchorage-independent growth. By contrast, hundreds of genes were not regulated in the same manner between the two lines, emphasizing the facts that 1) SVST targets additional B-type subunits and 2) SVST also

contains the extra N-terminal, PP2A-independent region which carries with it its own specific helper function. In fact, this study also examined the gene expression profile of HEKTER cells expressing an SVST mutant lacking the PP2A interacting domain (ST110) which revealed that over 100 genes were affected by SVST in a PP2A-independent manner when compared to full-length expression of SVST or knockdown of B'γ. These findings suggest that the PP2A-independent region of SVST, which cannot promote transformation alone, may contribute to transformation in conjunction with the suppression of SVST-targeted PP2A B-type subunits. However this potential role for the PP2A-independent region has yet to be determined. Together, the use and further study of this defined transformation cell line can provide unique insights to better understand how the interaction of SVST and PP2A promotes transformation and to gain insights into the process of oncogenic initiation and progression.

Polyomavirus

The study of Polyomavirus, like SV40, has also provided some key insights into mechanisms regulating cell proliferation and survival and has furthered our understanding of the process of transformation. Like SV40, the 5kb genome of Polyomavirus contains an early region which encodes three alternatively spliced transcripts encoding three different proteins, namely the Large Tumor antigen (LT), the Middle Tumor antigen (MT) and the Small Tumor antigen (ST). All three oncoproteins are involved in viral replication and transformation and share a common 79 amino acid N-terminus which possesses chaperone activity from its J domain. Polyomavirus MT (PyMT) and Polyomavirus ST (PyST) share an additional 112 amino acids and thereafter

contain unique C-terminal sequences [195]. Like SVST, PyMT and PyST mediate cellular transformation through association with the PP2A A subunit [173] at the B-type subunit binding site, specifically at HEAT repeats 2-8 [196], thereby displacing some B-type subunits. Although it would seem that PyMT and PyST would function much in the same manner as SVST due to sequence similarities and their ability to bind PP2A [173, 186, 197], there are key differences in how PyMT and PyST mediate signaling changes. For example, although PyMT or PyST can inhibit the formation of some PP2A complexes by replacing certain B-type subunits and thus block the function of those holoenzymes, these T antigens can also target other proteins and differentially regulate cellular processes like apoptosis [186, 195]. Although PyMT and PyST oncoproteins can bind to a number of proteins, for the purposes of this thesis, only the interactions with PP2A will be briefly discussed.

PyMT is, by far, the most essential T antigen in Polyomavirus-mediated transformation as it is not only required, but is also sufficient in many cases, for transformation and tumor development [198, 199]. PyMT-mediated transformation is dependent on PP2A binding [200] and mutations that specifically block PyMT association with PP2A completely inhibit transformation, highlighting the essential nature of this interaction [173, 195]. In one study, association of PyMT with PP2A substantially reduced (~40-fold) PP2A activity towards the histone H1 substrate [42] demonstrating that, like SVST, PyMT inhibits PP2A phosphatase activity and thus likely promotes transformation through this inhibition. PyMT has also been shown to bind other targets including c-Src in concert with PP2A [201-203] to promote PI3K, Shc and PLCγ recruitment to the PyMT-PP2A-Src complex to be activated [204-206], thus promoting

prosurvival signaling. In support of PyMT's role in transformation, expression of PyMT causes PP2A-dependent activation of the Akt [207, 208] and MAPK pathways [206, 209] similar to SVST. While the ability of PyMT to promote transformation is clearly established in multiple studies, the mechanisms of PyST are still being elucidated. The expression of PyST alone does not cause the same tumorigenic effects as PyMT but does compliment PyMT-mediated transformation [186]. Some studies have linked PyST expression to progression of the cell cycle [210-213] and activation of MAPK and Akt pathways [62, 209, 214], thus supporting progrowth, prosurvival signaling. Interestingly, the function of PyST can switch between pro-apoptotic to anti-apoptotic through modulation of Akt depending on nutrient conditions [62]. Specifically, PyST can prevent apoptosis in low nutrient conditions by enhancing phosphorylation of Akt T308 and S473 resulting in downstream phosphorylation and inhibition of pro-apoptotic FOXO proteins and BAD. Conversely, in 10% serum, PyST still promotes Akt T308 phosphorylation. However, the end result is the induction of apoptosis. Altogether, these findings emphasize the complexity of polyomavirus and reveal significant deficiencies in our understanding of how the virus modulates PP2A to promote transformation.

In general, the common feature of the DNA tumor viruses is to disrupt PP2A holoenzyme formation, although in different subcellular compartments. Published data, as well as unpublished data from the Pallas lab, suggest that SVST, PyST, and PyMT probably target many of the same B-type subunits. Considering that many of the PP2A complexes containing methylation-sensitive B-type subunits are disrupted by the abovementioned T antigens and the fact that these complexes function in controlling cell growth and survival, it would seem likely that the T antigens may target methylation-

sensitive B-type subunits as a general strategy for overcoming the negative regulatory actions of PP2A. Therefore, further research is needed to elucidate the nature of the subunits replaced to better understand PP2A's tumor suppressive function.

Overview and Goals of the Dissertation

In the past few decades, much research has focused on the therapeutic importance of kinases in disease models, including cancer, leading to the development of a large number of kinase-targeting drugs. Only recently has the significance of modulating cellular signaling and function by targeting protein phosphatases begun to be explored [1, 215]. Research has shown that inhibiting PP2A promotes transformation in mammalian cells, providing strong evidence that PP2A functions as a tumor suppressor [24, 200]. Furthermore, disruption of PP2A holoenzyme formation by viral T antigens (MT and ST) promotes transformation by blocking a specific subset of PP2A complexes, resulting in the activation of multiple progrowth and prosurvival signaling pathways [172, 173]. SVST replaces a portion of Bα, B'α, and B'γ , and possibly Bδ, from PP2A complexes [98, 171, 173, 175]. Interestingly, these B-type subunits appear to be "methylation-sensitive" [43, 44, 46, 52, 216], that is, their incorporation into PP2A heterotrimers is enhanced by methylation of PP2Ac (Figure 5). Whether this unifying feature (methylation-sensitivity) of the nature of the B-type subunits replaced represents a possible strategy that T antigens override to promote transformation remains to be determined. Furthermore, inhibition of B'γ alone in place of SVST, only partially recapitulates SVST-mediated transformation [171], suggesting that other B-type subunits may need to be replaced concomitantly with B'γ to achieve full transformation. However,

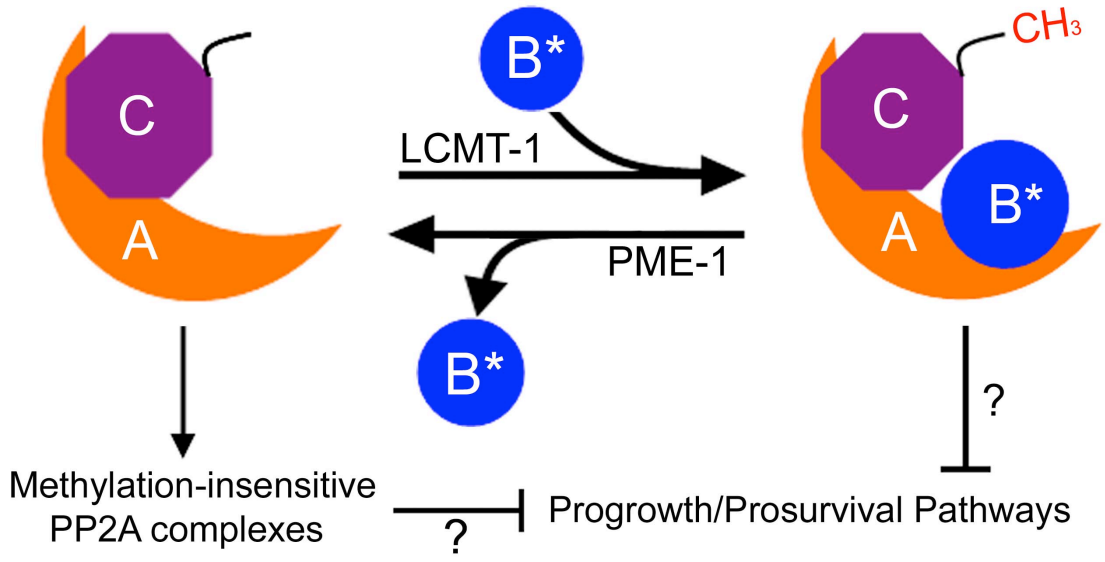


Figure 5. General model illustrating the regulation of PP2A complexes by PP2Ac methylation and PP2A's regulation of progrowth, prosurvival pathways. It was known that PP2A could be reversibly methylated by LCMT-1 and PME-1 and this modification could dictate the formation of specific PP2A complexes based on the B-type subunit binding. While some B-type subunits preferentially bind to the methylated form of PP2Ac (B*), other B-type subunits can bind regardless of the methylation status of PP2Ac (methylation-insensitive PP2A complexes). Furthermore, it was also known that PP2A functions as a tumor suppressor by inhibiting a number of progrowth, prosurvival pathways. However, at the beginning of my research, little information was available as to which PP2A complexes (dictated by the B-type subunits) could function as tumor suppressors and negatively regulate these progrowth, prosurvival pathways. Additionally, it was unknown whether the methylation status of PP2Ac (and therefore the assembly of methylation-sensitive heterotrimers) could play a role in directing PP2A's tumor suppressive function.

the transforming effect of suppressing additional B-type subunits in conjunction with B'γ knockdown has not been tested. Additionally, the N-terminal region of SVST contains a helper function and is linked to upregulating multiple progrowth, prosurvival functions [184]. Although this domain is not sufficient for transformation [178], it remains to be determined whether expressing this region will complement the B'γ knockdown to increase anchorage-independent growth.

To address these gaps in knowledge, the studies conducted in this thesis investigated how T antigens promote transformation by replacing a subset of PP2A regulatory B-type subunits and whether modulating PP2Ac methylation or methylation-sensitive PP2A complexes can contribute to transformation. In Chapter 3.1, I explored the hypothesis that circumventing normal cellular control of PP2A by methylation is a general strategy for ST- and MT-mediated transformation. To test the hypothesis, I assayed the binding of SVST and PyST to methylated and unmethylated PP2Ac and also examined the effects of reducing PP2Ac methylation on transformation and biochemical signaling pathways. The results from these experiments provided support for the idea that assembly of PP2A heterotrimers containing MT and ST oncoproteins is not regulated by PP2Ac methylation, whereas formation of at least some of the key PP2A heterotrimers they replace is regulated by the methylation status of PP2Ac. The results from Chapter 3.1 led me to explore the individual contributions of the N-terminal, PP2A-independent portion of SVST and of reducing methylation-sensitive PP2A complexes in conjunction with B'γ knockdown to transformation. The findings demonstrated mixed results on transformation which revealed that MT and ST-mediated transformation is quite complex and is likely modulated through the concomitant expression of the N-terminal domain

and reduction of multiple methylation-sensitive PP2A complexes. In Chapter 3.3, I investigated the effects on transformation of reducing PME-1. The findings revealed that enhancing PP2Ac methylation supports PP2A's tumor suppressive role. Overall, these studies provide multiple insights into a novel strategy that T antigens may utilize to overcome the negative regulatory actions of PP2A. The research here also provides further evidence for the involvement of PP2Ac methylation in supporting the tumor suppressive role of PP2A. Lastly, these findings imply that promoting PP2Ac methylation (e.g. PME-1 inhibitors) may serve as a novel therapeutic strategy to combat cancer.

CHAPTER 2

Methods and Materials

Cell Culture

HeLa and HEK293T cells were obtained from American Type Culture Collection (ATCC), QBI-HEK293 (QBiogene), HeLa-H2B-GFP [217], and HEKTERASB56γ [171] cells were kind gifts from E. Werner (Emory University), K.F. Sullivan (Scripps Research Institute), and W.C. Hahn (Dana Farber Cancer Institute), respectively. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20μM L-glutamine, 100U/mL penicillin G, 100μg/mL Streptomycin, and 250ng/mL amphotericin B and grown in 10% CO_2 at 37°C.

Transfection of shRNAs for Lentivirus Production

To create lentivirus expressing shRNAs, $5x10^6$ HEK293T cells, grown in normal media without antibiotics, were plated on 10 cm dishes and transfected the next day with either an empty vector (pLKO.1) or shRNA targeting one of the various PP2A-related genes listed in Table 1 (shRNAs acquired from The RNAi Consortium). Specifically, 3ug each of VSV-G (viral envelope plasmid), Δ8.9 (viral gag/pol/rev plasmid) and the shRNA plasmid and 0.3ug of pEGFP-N1 plasmid (transfection reporter) were transfected using 30uL FuGENE6 (Promega). FuGENE6 was preincubated with serum-free OptiMEM for 5 minutes prior to combining with premixed plasmids. Thereafter, transfection solution containing the plasmids, FuGENE and OptiMEM was incubated for 30 minutes at room temperature before adding to HEK293T transfection plates. Cells

were transfected overnight and then refed with 10mL target cell media the next day. At 36 hours, lentiviral supernatants were collected and filtered through a 0.45um syringe filter to remove any HEK293T cells. Supernatants were then treated with 5ug/ml polybrene (Hexadimethrine bromide) to assist in the virus' entry into target cells and then frozen and stored at -80°C.

Target	Symbol	TRC shRNA ID	shRNA Sequence
LCMT-1	L3	TRCN0000035061	5'-CGTCGACATGATGGAGTTGTA-3'
PME-1	P2	TRCN0000003058	5'-CTGGTGTTGATAGATTGGATA-3'
PME-1	H5	TRCN0000003060	5'-GCTTATCCAATCTCTTTCTTA-3'
PP2A Bα	B3	TRCN0000002493	5'-GCAAGTGGCAAGCGAAAGAAA-3'
PP2A Bδ	B10	TRCN0000063710	5'-GTCCTTCTTCTCAGAAATAAT-3'
PP2A Bδ	B12	TRCN0000063712	5'-CGGGTCCTATAACAACTTCTT-3'
PP2A B'α	H6	TRCN0000010508	5'-GAAGGAATTGGAACGTGAAGA-3'
PP2A B'α	H5	TRCN0000010507	5'-CACTGAATGAACTGGTTGAGT-3'

Table 1. shRNAs (acquired from The RNAi Consortium (TRC)) targeting PP2A B-type subunits, LCMT-1 and PME-1. Targets are listed with their corresponding TRCN IDs, target sequences, and the symbols used in this thesis.

Lentiviral Titering and Infections

Lentiviral titering to determine the concentration of viral particles in each lentiviral supernatant was necessary to infect HEKTERASB56γ cells due to the fact that these cells already contained the puromycin-resistant B'γ shRNA plasmid and could

therefore, not be further selected for expression of additional puromycin-resistant shRNAs. To circumvent this issue, HeLa cells which contain no puromycin resistance were used to determine the titer of each shRNA-expressing lentivirus. HeLa cells were plated at $5x10^4$ cells in 3.5 cm dishes in triplicate for each lentivirus being titered. The next day, HeLa cells were infected with 5uL, 25uL and 100uL of each virus overnight and then refed with normal media the next day. Two days post-infection, infected HeLa cells were fed media supplemented with 2ug/mL puromycin and allowed to grow until a canary dish which had not been infected was completely dead in response to puromycin treatment (approximately 3-5 days). HeLa cells successfully infected with the lentiviruses were collected and counted by hemacytometer. Total cells were divided by the average number of cells/colony to determine how many cells had been initially infected at day 1 and this number was then multiplied by the dilution factor of the virus to determine the number of viral particles per mL of lentivirus. The titer counts for all three dilutions were averaged to acquire the final concentration of viral particles/mL of virus.

To create HEKTERASB56γ cells with stable knockdowns, cells were plated at an appropriate concentration to be infected at a multiplicity of infection (MOI) of 3 using 0.5mL of virus. This infection rate would ensure that >95% of cells would receive at least 1 viral particle as determined by the Poisson distribution. Cells were also infected with vector control (pLKO.1) virus by the same process described here. HEKTERASB56γ cells containing vector control (VC) or the shRNAs were expanded, tested by western analysis for the knockdown efficiency, and frozen for future experiments. All resulting cell lines for this study represent populations of thousands of different infected cells and are not from individual clones.

Creation of the LCMT-1-Rescue cDNA Sequence.

To create an LCMT-1-rescue construct that expresses LCMT-1 mRNA resistant to the L3 LCMT-1 shRNA, four silent mutations (depicted here as upper case letters) were introduced by PCR into the LCMT-1 coding region targeted by the L3 LCMT-1 shRNA: 5' cgtcgaTatgatggaACtCta 3'. The entire cDNA was sequenced to ensure that only the intended bases were altered.

Creation of HEKTERASB56γ Cells That Stably Express Akt-AA, PME-1, ST110, or an shRNA-resistant LCMT-1.

*c*DNA sequences encoding dominant-interfering Akt-AA (Akt T308A/S473A; gift from Dr. Wei Zhou) [218], PME-1, or L3 shRNA-resistant LCMT-1 were cloned into the blasticidin-resistant pLenti6-V5-D-Topo vector using conventional cloning techniques or ligation independent cloning. SV40 small T antigen mutant, ST110, was made by creating PCR primers to introduce a stop codon at amino acid 111. The primers also introduced restriction sites on either side of ST110 to facilitate its ligation into the pLenti6-V5-D-Topo vector backbone. Lentiviruses expressing these proteins were then generated using Invitrogen's ViraPower® 4-plasmid lentiviral system and viral titers were determined as described above. For LCMT-1 overexpression and rescue lines, HEKTERASB56γ-VC or -L3 knockdown cells were infected with the LCMT-1-rescue lentivirus creating LCMT-1 OE and L3-rescue cells, respectively. Similarly, Akt-AA was expressed in both the VC and L3 knockdown cells creating VC-Akt-AA and L3-Akt-AA, respectively. HEKTERASB56γ cells were infected with the PME-1 lentiviruses or ST110 to create HEKTERASB56γ-PME-1 OE and HEKTERASB56γ-ST110, respectively. For

all lines, vector control cell lines were created by infection of the same cells with lentiviruses containing an empty pLenti6-V5-D-Topo plasmid. Cells were infected overnight, selected with 7.5ug/mL blasticidin S-HCl two days post-infection, tested by western analysis for protein expression of the expressed plasmid, and frozen for future experiments. All resulting cell lines for this study represent populations of thousands of different infected cells and are not from individual clones.

Antibodies

LCMT-1 was detected using an affinity-purified rabbit anti-LCMT-1 polyclonal antibody, RK3110, described previously [53]. Other antibodies used included sepharose bead-conjugated anti-HA-tag antibody used for immunoprecipitation (F-7 AC; Santa Cruz), anti-HA antibody used for western blotting (16B12; Covance), anti-GFP mouse monoclonal antibody (B-2; Santa Cruz), anti-SVST rabbit polyclonal antibody (gift from W. Hahn), anti-PyST rabbit polyclonal antibody [219], PP2A Bα mouse monoclonal antibody (clone 2G9; Millipore), PP2A B'α rabbit polyclonal antibody (Bethyl Laboratories), PP2Ac mouse monoclonal antibody (BD Transduction Laboratories), unmethylated PP2A C subunit mouse monoclonal antibody (clone 1D6 [43]; available from Millipore, Santa Cruz Biotechnology, or request to the Pallas lab), p-Akt T308 and p-Akt S473 rabbit monoclonal antibodies (Epitomics), GAPDH mouse monoclonal antibody (Abmart), Pme1 mouse monoclonal antibody (B12; Santa Cruz Biotechnology), mouse monoclonal antibodies to p-S6K (p70 T389 and p85 T412) and total rpS6, and rabbit polyclonal antibodies to p-GSK3B S9, p-rpS6 S235/236, total Akt, total p70/p85

S6K, and pAkt substrate ((R/K)X(R/K)XX(pT/pS)) obtained from Cell Signaling Technology.

Cell Lysis and Western Analysis

For cell lysis, cells were washed once each with ice-cold phosphate-buffered saline (PBS) and IP wash buffer containing 0.135M NaCl, 20nM Tris-pH 8.0, and 10% glycerol. Cells were then incubated with IP lysis buffer (IP wash buffer plus 1% Nonidet P-40, 0.04 trypsin inhibitor units/mL aprotinin, 1mM phenylmethylsufonyl fluoride (PMSF), and 50mM sodium fluoride (NaF)) for 20 minutes while rocking at 4°C. Cells were then further lysed by passing through a 25 gauge needle and then cleared of debris by centrifugation at 13,000 x g. For western analysis, samples containing equal amounts of protein as determined by Lowry protein assay (Bio-Rad) were analyzed on SDS-polyacrylamide gels. All experiments were conducted using 10% SDS-PAGE gels unless otherwise stated in figure legends. Gels were transferred onto nitrocellulose membranes and blocked in 5% non-fat milk/Tris-Buffered Saline-0.1% Tween 20 (TBS-T) solution at room temperature for 1 hour. Thereafter, membranes were incubated with primary antibodies in 1% non-fat milk/TBS-T solution overnight in a 4°C hybridization incubator (Techne). The next day, membranes were washed with TBS-T and incubated with secondary antibodies coupled to horse-radish peroxidase at room temperature for 1 hour. After secondary antibody incubation, membranes were again washed and protein bands were visualized by enhanced chemiluminescence using a BioRad Fluor-S Max Chemilumimager equipped with a supercooled CCD camera which measures band intensities and provides linear data over 4.8 orders of magnitude. Quantity One® software

was used to quantify band intensities. Statistical significance was established by Student's t-tests where P values ≤ 0.05 determine the threshold for significance. *, P≤0.05; ** P≤0.01.

Transfection Procedure for Expression of SVST, PyST and PP2Ac

For SVST and PyST binding experiments, $5x10^6$ QBI-HEK293 cells were plated onto 6 cm tissue culture dishes. The next day, cells were cotransfected as described above, using 9uL FuGENE, with either 1.5ug SVST or PyST and 1.5ug pcDNA3.1 HA-wild-type PP2Ac, pcDNA3.1 HA-D85N PP2Ac, or pcDNA3.1 HA-L309Δ PP2Ac. The two PP2Ac mutants have previously been used to distinguish between methylation-sensitive and methylation-insensitive PP2A heterotrimer formation. Specifically, L309Δ is a PP2Ac mutant lacking the methylated carboxyl-terminal L309, and D85N is an active site PP2Ac mutant with an intact, but unmethylated L309 [44, 216]. One dish per experiment was also transfected with 3ug pcDNA3.1 empty vector as a control. Additionally, all plates were cotransfected with a pEGFP-N1 plasmid as a readout of transfection efficiency. Cells were transfected overnight and then refed with normal media. Two days post-transfection, cells were lysed and prepared for immunoprecipitation experiments.

Immunoprecipitation

To immunoprecipitate HA-tagged proteins and complexes, cells were lysed as described above and then 90% of the lysates were incubated with 40uL of sepharose bead-conjugated anti-HA antibody (F-7 AC) for 1.5h while rocking at 4°C. After

washing twice with PBS and lysis buffer, 30uL of a 1:4 dilution of GS (33% glycerol/6.7% SDS) gel loading buffer:IP lysis solution was added to the bead complexes and then boiled for 2 minutes to separate the immune complexes from the sepharose beads. The mixture was centrifuged and the supernatant was collected which contained the immune complexes of interest. HA epitope-tag immunoprecipitates were then treated with 6uL of reducing agent (100mM DTT), boiled, and then resolved by 12% SDS-PAGE. The 10% of lysates not immunoprecipitated was also loaded to confirm the expression of the plasmid proteins. Gels were transferred and Western blot analysis was conducted as described above. Assays were done at least three times to ensure the continuity of the results.

PP2A C Subunit Methylation Assay.

The steady-state level of PP2Ac methylation in lysates was determined using a monoclonal antibody (clone 1D6 [7]; Millipore, Santa Cruz Biotechnology, Dr D. Pallas) specific for unmethylated PP2Ac. Cell lines were lysed as described above except that the lysis buffer was supplemented with 250nM of okadaic acid to inhibit additional PP2Ac methylation or demethylation which might occur during the lysis procedure. Cells were lysed in 40uL lysis buffer and exactly half of each lysate (20uL) was treated for 5 minutes on ice with 20uL of 200mM NaOH to completely demethylate PP2Ac and then neutralized with 30uL of neutralization buffer (133.3mM HCl and 333.3mM Tris pH 6.8). The other half of the lysates (20uL) not subjected to base treatment were treated with 50uL preneutralized base solution (80mM NaOH, 80 mM HCl, and 200 mM Tris pH 6.8) as a readout for total endogenous demethylated PP2Ac. Samples were then subjected

to 10% SDS-PAGE and immunoblotted with the methylation-sensitive 1D6 monoclonal antibody which detects unmethylated PP2Ac and α-tubulin as a loading control. Signals were detected and quantitative analysis was performed as described in the Cell Lysis and Western Analysis section above. The percent unmethylated PP2Ac was determined by quantitatively comparing the unmethylated PP2Ac signals in the untreated (–) lanes and base-treated (+) lanes. Percent methylated PP2Ac was calculated by subtracting percent unmethylated PP2Ac from 100. Assays were done at least three times. Statistical significance was established by Student's t-tests where P values ≤ 0.05 determine the threshold for significance. *, $P \leq 0.05$; ** $P \leq 0.01$.

Anchorage-Independent Growth (Soft Agar Analysis)

Anchorage-independent growth was assessed by soft agar analysis. Agars consist of two different layers, a 0.6% agar bottom layer and a less dense, 0.3% top layer which contains the cells of interest. To make the bottom agar layer, sterilized 2.4% noble agar was melted and warmed to 42°C in a water bath. Thereafter, the liquid agar was added at a 1:4 ratio to 2X DMEM media supplemented with 10% FBS, 20μM L-glutamine, 100U/mL penicillin G, 100μg/mL Streptomycin, 250ng/mL amphotericin B, and sterile water to make a final 1X DMEM final concentration, and plated on 3.5 cm dishes at 2mL/plate. Bottom agars solidified within 10 minutes at room temperature. Before making the top agar layer, cells were trypsinized and counted by hemacytometer to acquire $5x10^3$ cells/agar. To make the top layer, the 42°C, sterilized 2.4% noble agar (temperatures in excess of 42°C may cause cell death) was added at a 1:8 ratio to the 2X DMEM media described above. 2ml of the 0.3% agar-media mixture was then added to

the $5x10^3$ cells and plated on top of the 0.6% bottom agar. Top agars required at least 20-25 minutes to solidify at room temperature. Agars were incubated in 10% CO_2 at 37°C for three weeks and fed with 0.25mL of 10% FBS/DMEM weekly to maintain moisture and nutrients. After three weeks of growth, agars were photographed and quantitatively analyzed for changes in colony number and size. For colony image figures, small, representative, microscopic fields within the dishes were photographed at 40x using an Olympus IX81 microscope equipped with a Nikon Fi1/U2 color camera and processed using NIS Elements software. Colonies were scored for number and volume using a standard Olympus CK2 benchtop microscope equipped with an eyepiece micrometer reticle. Colonies 0.1 mm in diameter or larger were counted. All assays were performed three times in triplicate unless otherwise specified in the figure legend. For all agar experiments, statistical significance was established by Student's t-tests. *, $P \leq 0.05$; ** $P \leq 0.01$.

Suspension Cultures.

To analyze biochemical changes in anchorage-independent cells, cells were plated onto Corning 100 mm ultra low attachment culture dishes at $5x10^5$ cells/plate and grown for 1 week in DMEM supplemented with 10% FBS. The cells formed spherical colonies similar to those seen in soft agar although, due to the lack of matrix, colonies were not stationary and were sometimes interconnected. Because of this, and the fact that attempts to totally dissociate the cells to obtain counts resulted in substantial cell lysis, I used total cell weights as a measure of growth. After 1 week in culture, colonies were collected,

washed, pelleted, and then all excess fluid was removed and the cell pellets were weighed on a highly sensitive scale. Cells were then lysed and analyzed as described above.

Anchorage-Dependent Growth Assays

Four-day anchorage-dependent growth rates were assessed by plating $2.5x10^4$ cells in triplicate on 3.5 cm plates on day 0 and collecting and counting all viable cells (adherent and detached) at Days 1, 2, and 4. Seven-day anchorage-dependent growth rates were assessed by plating $1x10^4$ cells on 10 cm dishes on day 0 and collecting and counting all viable cells (adherent and detached) at days 1, 3 and 7. Cells were stained with trypan blue to exclude dead cells and counted by hemacytometer. All assays were performed at least three times.

Anchorage-Dependent Death Assays

To assess death rates, cells were plated at $5x10^4$ cells per 3.5 cm tissue culture dish in triplicate and media was changed the next day. After 3 days in culture, both adherent and detached cells were collected, stained with trypan blue to identify dead cells and counted by hemacytometer. The percent of total dead cells was calculated by dividing the total number of trypan blue positive cells by the total number of cells (+ and - trypan blue). Similarly, death rates in low serum were assessed by plating $5x10^4$ cells per 3.5 cm tissue culture dish in triplicate. After 24 hours, cells were fed with a 0.1% FBS-containing media (low serum) and grown for an additional 48 hours. Cells were then treated as described above and percent cell death was determined. All assays were performed at least three times.

Time-Lapse Microscopy

For Bδ knockdown time-lapse experiments in HeLa cells, cells were plated at appropriate concentrations as determined by lentiviral titering of the vector control (VC) virus, the B10 Bδ-targeting virus and the B12 Bδ-targeting virus and infected overnight at a MOI of 3 the following day. After overnight infection, HeLa cells were refed with normal media and allowed to recover and grow for an additional day before conducting experiments. Forty-eight hours post-infection, infected HeLa cells were then incubated at 37°C in an atmosphere and temperature-controlled stage incubator (M6; Zeiss Instruments). An Olympus IX81 phase/fluorescence microscope equipped with an ASI computer-driven motorized stage was used to conduct time-lapse experiments. Phase images were captured over a 4 day period at 6 minute intervals and analyzed using Slidebook Microscope Analysis software (Intelligent Imaging). For Bδ knockdown or SVST expression time-lapse experiments, fluorescence images were captured by a filtered fluorescence illuminator. For these experiments, cells were observed for 60-72 hours at 6 minute intervals and images were analyzed by the aforementioned software.

CHAPTER 3.1

Results

Circumventing Cellular Control of PP2A by Methylation Promotes Transformation in an Akt-dependent Manner

(text and figures in Chapter 3.1 are adjusted from published manuscript: Jackson, J.B., Pallas, D.C. (2012) Neoplasia 14(7):585-599. See reference [220])

Hypothesis: Circumventing normal cellular control of PP2A by PP2Ac methylation is a general strategy for ST- and MT-mediated transformation.

Predictions: 1) PyST and SVST, like PyMT, will bind PP2A in a "methylation-insensitive" manner and 2) down-regulation of PP2Ac methylation will activate progrowth, prosurvival signaling and promote transformation.

SVST and PyST Do Not Require PP2Ac Methylation for PP2A Heterotrimer Formation

I hypothesized that replacement of methylation-sensitive cellular B-type subunits by methylation-insensitive viral B-type subunits (MT and ST) is an important strategy of polyomavirus and SV40 for circumventing normal control of cell growth and survival during transformation (Figure 6A). To test this hypothesis, I first determined whether SVST and PyST, like PyMT, form PP2A heterotrimers independently of PP2Ac methylation. For these experiments, I used two PP2Ac mutants that had previously been

used to distinguish between methylation-sensitive and methylation-insensitive PP2A heterotrimer formation: L309Δ, a PP2Ac mutant lacking the methylated carboxyl-terminal L309, and D85N, an active site PP2Ac mutant with an intact, but unmethylated L309 [44, 216]. Of note, the latter mutant indirectly inhibits methylation presumably by blocking association with LCMT-1 (mimicking an LCMT-1 knockdown effect) [54]. I tested whether SVST and PyST could form heterotrimers containing the L309Δ and D85N mutants by using coimmunoprecipitation. SVST and PyST coimmunoprecipitated to the same extent with wild-type, L309Δ, and D85N PP2Ac (Figure 6B and C), whereas endogenous methylation-sensitive Bα only coimmunoprecipitated with wild-type PP2Ac. This result supports the idea that assembly of PP2A heterotrimers containing MT and ST oncoproteins is not regulated by PP2Ac methylation, whereas formation of at least some of the key PP2A heterotrimers they replace is regulated by the methylation status of PP2Ac.

Loss of LCMT-1 Promotes Transformation

A second prediction of my hypothesis is that reducing PP2Ac methylation by decreasing the level of the methyltransferase responsible for PP2Ac methylation, LCMT-1, would promote transformation. To test this prediction, I used a genetically defined transformation system in which immortalized primary human embryonic kidney cells expressing SV40 large tumor antigen, activated H-Ras, and human telomerase (HEKTER cells [178]) have been weakly transformed by down-regulation of the PP2A B-type subunit, B56γ (HEKTERASB56γ cells [171]; see Figure 4 in Introduction). Whereas HEKTER cells can grow indefinitely in culture, they form very few anchorage-

independent colonies in soft agar [171, 178]. When SVST is expressed in the immortalized HEKTER line, the cells grow robustly in soft agar. Knocking down B56γ, a target of SVST, in HEKTER cells partially recapitulates SVST-mediated transformation phenotype [171]. This weakly transformed HEKTERASB56γ line was utilized for the following studies because it would allow for the determination of the cumulative effect of targeting additional methylation sensitive B-type subunits and to detect either increased or decreased transformation upon LCMT-1 down-regulation. HEKTERASB56γ cells were infected with vector control (VC) or LCMT-1-directed (L3) shRNA virus, and Western blot analysis was used to verify the knockdown of LCMT-1 (Figure 7A). Quantitation of three independent experiments showed that the LCMT-1 shRNA stably reduced LCMT-1 expression by 80% ± 6% compared with the VC cells. In addition, LCMT-1 knockdown reduced the steady-state level of the methylation-sensitive PP2A Bα subunit (Figure 7A) by 28% ± 15% (P =.03), showing that the LCMT-1 knockdown affects at least one B-type subunit that depends on LCMT-1 for heterotrimer formation.

Next, I measured the effect of LCMT-1 knockdown on the steady-state level of PP2Ac methylation using an assay employing a monoclonal antibody specific for unmethylated PP2Ac (Figure 7B). As can be seen by comparing the minus base (-) lanes (which show endogenous levels of unmethylated PP2Ac) in Figure 7B, the amount of unmethylated PP2Ac greatly increased in L3 LCMT-1 knockdown cells compared with the VC control cells. Quantitation of the results showed that only 17% of the total PP2Ac in the LCMT-1 knockdown cells remained methylated compared to 74% methylation in the control cells (Figure 7C). Thus, LCMT-1 knockdown reduced steady-state PP2Ac methylation by more than four-fold.

To examine the role of LCMT-1 and PP2Ac methylation in transformation, VC and L3 cells were evaluated for anchorage-independent growth in soft agar, an assay highly predictive of tumorigenicity [221]. After 3 weeks of growth in agar, colonies were examined by microscopy, revealing that L3 cells formed larger and more abundant colonies (Figure 7D). Quantitative analysis showed that L3 cells had a two-fold increase in colony number (Figure 7E) and more than a four-fold increase in colony volume compared to VC cells (Figure 7F). These results strongly support the hypothesis that LCMT-1 is a negative regulator of transformation and are consistent with the idea that circumvention of PP2Ac methylation-regulated control of PP2A function contributes to MT and ST-mediated transformation.

LCMT-1 Knockdown Does Not Affect Cell Size, Proliferation Rate, Survival, or the Akt and p70/p85 S6K Signaling Pathways during Normal Anchorage-Dependent Growth

To begin to dissect how a reduction in LCMT-1 promotes anchorage-independent growth, I tested whether the increase in colony growth of LCMT-1 knockdown cells in soft agar reflects changes in cell size, cell growth, and/or death rates in standard, anchorage-dependent tissue culture. VC and L3 cells growing in anchorage-dependent conditions were analyzed for forward scatter-area (FSC-A) by flow cytometry, which is a function of relative cell size. In three independent experiments, FSC-A was not substantially different (Figure 8A), supporting the conclusion that an increase in cell size on LCMT-1 knockdown does not account for the increase in the number and size of L3 colonies in soft agar. Comparison of the proliferation rates of VC and L3 cells over a 1-week period also showed no significant differences between the two cell lines, indicating

that LCMT-1 knockdown does not increase cell proliferation in anchorage-dependent conditions (Figure 8B). Quantitation of the amount of cell death in VC and L3 cultures during adherent cell growth also revealed no significant difference between the cell lines (Figure 8C). Similarly, no significant difference in cell death was seen when the VC and L3 cells were exposed to low serum conditions (0.1% FBS) for 48 hours to stress the cells (Figure 8D). Thus, under both normal and serum-starved growth conditions, no survival advantage is seen for LCMT-1 knockdown cells compared with control cells in anchorage-dependent growth conditions.

To assess whether LCMT-1 knockdown activates progrowth/prosurvival signaling pathways during anchorage-dependent growth, I compared the levels of regulatory phosphorylations on Akt, the Akt substrate, GSK3β, p70/p85 S6K, and ribosomal protein S6 (rpS6) in adherent cells. No significant differences in these phosphorylations were detected between the VC and L3 cells (Figure 8E-G). These results indicate that the activation state of these signaling molecules is not altered by LCMT-1 knockdown in adherent cultures, consistent with the lack of effect of LCMT-1 knockdown on proliferation and survival during normal anchorage-dependent growth.

LCMT-1 Knockdown Activates the Akt and p70/p85 S6K Pathways in Anchorage-Independent Conditions

The observation that LCMT-1 knockdown increased colony number and size in soft agar but had no significant effect on growth or survival in anchorage-dependent conditions raised the intriguing possibility that LCMT-1 knockdown might enhance growth and survival signaling exclusively during anchorage-independent cell growth.

When anchorage-dependent cells are unable to attach to an appropriate substratum, reduced growth and survival signaling normally occurs, resulting in cessation of cell proliferation and eventually cell death [222]. I hypothesized that LCMT-1 is necessary for the down-regulation of key proliferation and survival signals in anchorage-independent conditions and that LCMT-1 knockdown inappropriately maintains elevated levels of progrowth and prosurvival signaling during anchorage-independent growth. To test this hypothesis, I analyzed the Akt and p70/p85 S6K signaling pathways in VC and L3 cells growing in anchorage-independent conditions on ultra-low-adherence tissue culture dishes. In these dishes, both the VC and L3 cell lines proliferate and form spherical, anchorage-independent clusters of cells. Similar to results from soft agar growth, the LCMT-1 knockdown cells showed a selective advantage over the control cells as indicated by a 70% increased total weight of the L3 cells compared with the VC cells after 1 week of growth under these conditions (Figure 9A). Considering the difference in assay time, I would expect that the L3 suspension colonies would have had a similar growth differential to the VC line as seen in soft agar if they were grown for 3 weeks in suspension cultures. Western blot analysis of the VC and L3 cell suspension cultures revealed a number of progrowth and prosurvival signaling differences. Relative to VC cells, L3 cells have increased activated Akt as indicated by increased phosphorylation at both T308, which is located in the kinase domain, and S473, which is located in the C-terminal regulatory domain (Figure 9B and C). Phosphorylation of these two sites is carried out by different kinases, and while phosphorylation of T308 partially activates Akt, full activation requires phosphorylation at both sites [59]. Furthermore, a downstream target of the Akt cell growth and survival pathway, GSK3β, showed

significantly increased phosphorylation on Ser9 in L3 cells (Figure 9B and C). Phosphorylation of Ser9 on GSK3β is inhibitory and would thus be expected to reduce the reported proapoptotic activities of GSK3β [77]. To further examine activation of Akt, I probed lysates from these cells with a phospho-Akt substrate antibody that specifically recognizes the (R/K)X(R/K)XX(pT/pS) motif when it is phosphorylated on the serine/threonine residue. Consistent with the finding of increased activated Akt on LCMT-1 knockdown, this antibody detected substantial increases in the phosphorylation of a number of proteins in L3 cells compared to VC cells (Figure 9D).

The (R/K)X(R/K)XX(pT/pS) substrate phosphorylation motif is also shared with S6K; therefore, some of the proteins with increased phosphorylation in the L3 lane of Figure 9D could be p70/p85 S6K substrates. For example, the bracketed protein of ~30 kDa in Figure 9D is likely rpS6, which is a substrate for p70 S6K but not Akt. Probing for activating phosphorylations on p70/p85 S6K showed that these kinases are indeed activated by LCMT-1 knockdown (Figure 9E and G). Immunoblot analysis with phospho-rpS6 and total rpS6 antibodies also confirmed that LCMT-1 knockdown increases rpS6 phosphorylation on a site that correlates with increased protein translation [223, 224] (Figure 9F and G). These results support the idea that LCMT-1 knockdown increases survival in an anchorage-independent environment through modulation of the Akt and p70/p85 S6K pathways.

Effects of LCMT-1 Knockdown on Growth and Signaling Are Rescued by Exogenous Expression of LCMT-1

To confirm that the effects of enhanced transformation and altered signaling in the LCMT-1 knockdown line are specifically due to decreased LCMT-1, I tested whether the effects of LCMT-1 knockdown could be rescued by exogenously expressing LCMT-1. To accomplish this, the original VC cells were infected with an empty expression vector (creating VC-control cells) and the original LCMT-1 knockdown cells (L3) were infected with either an empty vector (creating L3-control cells) or a vector expressing an L3 shRNA-resistant LCMT-1 mRNA (creating L3-rescue cells). Western blot analysis verified that LCMT-1 remained knocked down in the L3-control line and that LCMT-1 protein expression was rescued in the L3-rescue line (Figure 10A). Analysis of the level of PP2Ac methylation in these cell lines demonstrated that restoration of LCMT-1 expression restored the normal level of PP2Ac methylation in the L3-rescue cells (Figure 10B and C). To examine the ability of exogenous LCMT-1 expression to rescue the effect of LCMT-1 knockdown on transformation, VC-control, L3-control, and L3-rescue lines were subjected to soft agar analysis. The number of colonies produced by the L3-rescue cells was reduced almost three-fold from the L3-control cells and was comparable to the VC-control cells (Figure 10D and E), indicating that loss of LCMT-1 was indeed responsible for the increase in colony number observed on LCMT-1 knockdown. LCMT-1 reexpression (L3-rescue) also significantly reversed colony size compared to the LCMT-1 knockdown cells (L3-control; Figure 10D and F). These results support the conclusion that reduction of LCMT-1 enhances transformation in the HEKTERASB56γ cell line, presumably by hampering PP2A's tumor suppressor function. Consistent with

the hypothesis that LCMT-1 has a tumor suppressor function, overexpression (OE) of LCMT-1 in HEKTERASB56γ cells (Figure 11A) also substantially reduced colony number by 95% (Figures 11B and C) and volume by 58% in soft agar (Figures 11B and D). These differences in anchorage-independent growth were not reflected in anchorage-dependent proliferation rates (Figure 11E); therefore, the anti-transformative effects of PP2Ac methylation are specific to anchorage-independent cell populations. Furthermore, suspension cell lysates show that LCMT-1 overexpression significantly reduces Akt T308 phosphorylation by 22% ± 4% ($P<0.001$) and S473 phosphorylation by 27% ± 15% ($P=0.03$) (Figure 11F) as well as phosphorylation of some downstream Akt substrates (Figure 11G, arrows). Together, these data argue that loss of PP2Ac methylation promotes transformation, whereas increasing PP2A methylation reduces transformation.

To determine whether reexpression of LCMT-1 in L3 cells reverses the changes in the Akt and p70/p85 S6K signaling pathways induced by LCMT-1 knockdown, the VC-control, L3-control, and L3-rescue cells were grown in suspension in ultra-low-adherent culture dishes. Reexpression of LCMT-1 completely reversed the anchorage-independent growth advantage observed in L3-rescue cells (Figure 12A). Correspondingly, in suspension cell lysates the phosphorylation levels of T308 and S473 on Akt and of S9 on GSK3β were fully reversed to control levels (Figure 12B and C; compare L3 rescue to VC-control). Levels of phosphorylation on (R/K)X(R/K) XX(pT/pS) motif-containing proteins were also reversed to control levels (Figure 12D). Lastly, Western blot analysis revealed that reexpression of LCMT-1 fully reversed the activation of p70/p85 S6K and rpS6 phosphorylation (Figure 12E-G). These results demonstrate that the signaling changes detected in this system upon LCMT-1 knockdown

are due specifically to reduction in the LCMT-1 protein level. They also support the hypothesis that that loss of PP2A methylation promotes transformation, although I cannot rule out a possible contribution of a yet to be discovered LCMT-1 target.

Akt Activation Is Necessary for the Enhanced Transformation Caused by LCMT-1 Knockdown

To test the hypothesis that LCMT-1 knockdown increases transformation through activating the Akt survival pathway, I expressed a dominant-negative Akt (Akt-AA) in LCMT-1 (L3) knockdown cells (creating L3-Akt-AA cells). Akt-AA was also expressed in vector control cells (creating VC-Akt-AA cells) to assess the effects of dominant-negative Akt independent of LCMT-1 knockdown. VC-control, VC-Akt-AA, L3-control, and L3-Akt-AA cell lines were analyzed by Western blot to confirm the expression of the mutant Akt construct and the maintenance of LCMT-1 knockdown in L3-Akt-AA cells (Figure 13A). These lines were then subjected to soft agar analysis to assay for anchorage-independent growth (Figure 13B). The expression of Akt-AA completely reversed the increase in colony number (~2.4-fold) observed upon LCMT-1 knockdown and reduced the average colony volume of LCMT-1 knockdown cells by more than 2.3-fold (Figure 13C-D). In contrast, the expression of Akt-AA in HEKTERASB56γ VC cells resulted in only a ~30% reduction in soft agar colony number that was not statistically significant and reduced colony size by only 6% in soft agar (Figure 13C-D), indicating that the effect of expressing the kinase-deficient Akt in L3 knockdown cells is largely specific for LCMT-1.

To examine the effect of dominant-negative Akt-AA expression on Akt signaling, VC-control, L3-control, and L3-Akt-AA cells were grown in suspension and analyzed by Western blot. Because the VC-Akt-AA cell line resulted in little change in anchorage-independent growth, it was excluded from further analysis. Akt S473 phosphorylation and GSK3β S9 phosphorylation were reduced to control levels, whereas Akt T308 phosphorylation was partially reduced but not significantly (Figure 14A and B). Immunoblot analysis with the (R/K)X(R/K)XX(pT/pS) motif antibody showed that phosphorylation of most proteins affected by LCMT-1 knockdown was also reversed to control levels (Figure 14C). Lastly, I probed lysates from these lines for p70/p85 S6K activating phosphorylations and phosphorylation of rpS6. p70/p85 S6K activating phosphorylations were substantially and significantly reduced in L3-Akt-AA cells as was rpS6 phosphorylation (Figure 14D-F). Together, these results show that Akt signaling is required for the enhanced transformation caused by LCMT-1 knockdown and for much of the p70/p85 S6K pathway activation.

Overexpression of the PP2Ac Methylesterase, PME-1, Causes Similar Changes in Anchorage-Independent Growth and Signaling As LCMT-1 Knockdown

PME-1 overexpression provides another approach to test the effects of reduced PP2A methylation on transformation. To determine whether PME-1 overexpression would cause the same effects as LCMT-1 knockdown, I overexpressed PME-1 in HEKTERASB56γ cells (creating PME-1 OE cells). VC and PME-1 OE cells were analyzed by Western blot analysis to verify PME-1 expression (Figure 15A). PP2Ac methylation in cell lysates was reduced from 81% for VC cells to 26% for PME-1 OE

cells (Figure 15B-C). Results of experiments evaluating anchorage-independent growth in soft agar showed that PME-1 overexpression significantly increases colony number, but not colony volume (Figure 15D-F). To assess signaling changes in anchorage-independent conditions, cells were grown in suspension for 1 week, weighed, and lysed. Although no significant growth advantage was seen as measured by total suspension cell weight (Figure 16A), this may reflect the fact that PME-1 OE resulted in a modest increase in soft agar growth. Western blot analysis of VC and PME-1 OE suspension cell lysates for changes in the Akt and p70/p85 S6K pathways showed that PME-1 overexpression significantly increased T308 phosphorylation on Akt but not S473 phosphorylation on Akt or pGSK3β S9 phosphorylation (Figure 16B-C). Probing lysates from VC and PME-1 OE cells with (R/K)X(R/K)XX(pT/pS) substrate motif antibody demonstrated that PME-1 overexpression increased the phosphorylation of several proteins migrating at positions of proteins whose phosphorylation was increased in LCMT-1 knockdown cells (compare Figure 16D to Figure 9D). Lastly, activating phosphorylation of p70/p85 S6K and phosphorylation of rpS6 were significantly increased (Figure 16E-G). These results show that overexpression of PME-1 reduces PP2Ac methylation, increases transformation, and alters Akt and p70/p85 S6K signaling in a manner similar to LCMT-1 knockdown. Therefore, PP2Ac methylation, and thus the binding of methylation-dependent B-type subunits to PP2A, is important for PP2A's tumor suppressor function.

Together, these results clearly demonstrate that ST and MT antigens bind PP2A in a methylation-independent manner and that circumventing methylation partially mimics ST- and MT-mediated transformation of human cells. To further investigate how T

antigens promote transformation by replacing PP2A B-type subunits, in Chapter 3.2, I explore the transformative effects of knocking down the methylation-dependent B-type subunits targeted by the T antigens in conjunction with B'γ knockdown. Furthermore, I investigate the potential contribution of the PP2A-independent region of SVST to transformation to determine if this region can complement B'γ knockdown to further enhance transformation. Finally, results presented in this section imply that promoting PP2Ac methylation may serve as a novel therapeutic strategy for cancer. Therefore, in Chapter 3.3, I explore this hypothesis by knocking down the PP2A methylesterase, PME-1, to enhance PP2Ac methylation and analyze the effects on transformation in the HEKTERASB56γ system.

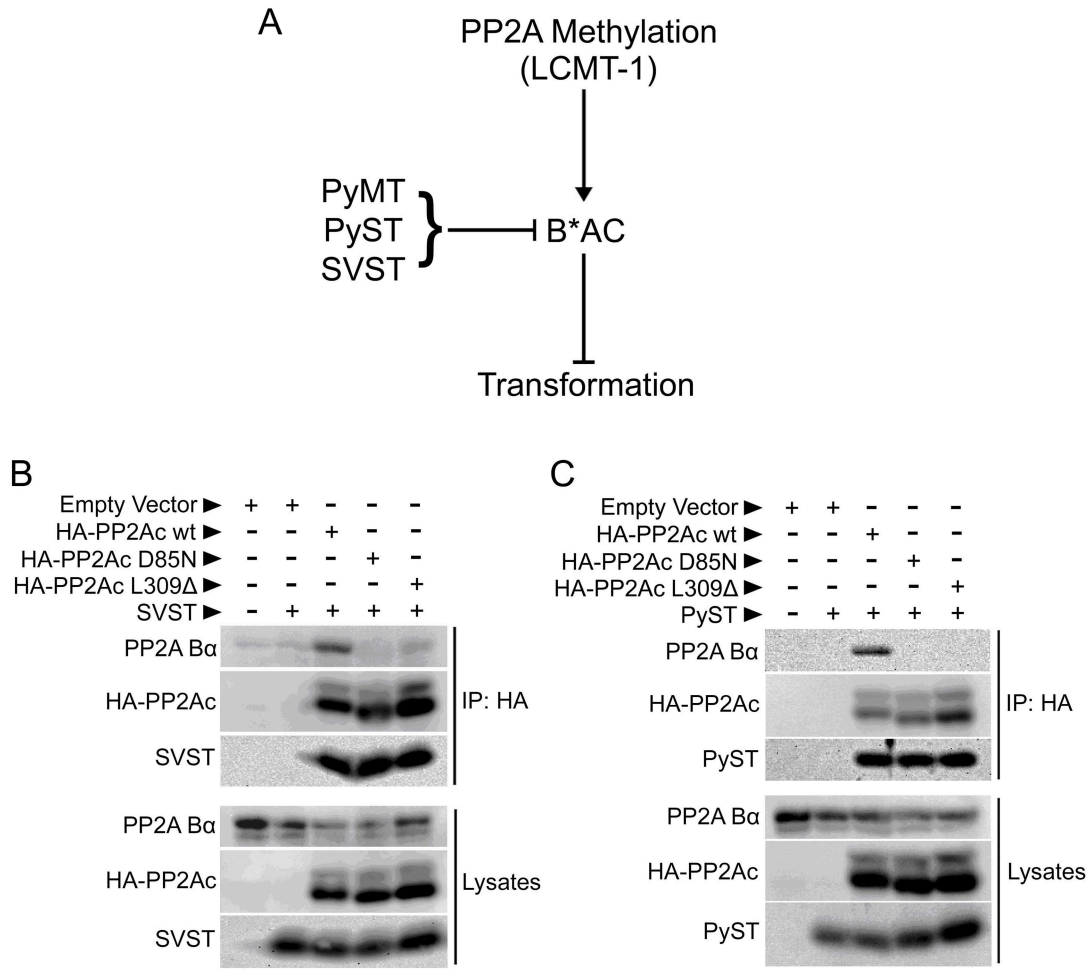


Figure 6. Unlike the methylation-dependent cellular B-type subunit, Bα, Simian Virus 40 small T (SVST) and Polyomavirus ST (PyST) can incorporate into PP2A heterotrimers independently of PP2Ac carboxyl methylation. (A) The diagram illustrates a potential strategy of polyomavirus and SV40 in which methylation-insensitive viral B-type subunits (PyMT, PyST, and SVST) specifically replace methylation-sensitive cellular B-type subunits (B*), thus promoting transformation by circumventing normal control of PP2A by methylation. LCMT-1 promotes PP2Ac subunit methylation and the assembly of methylation-sensitive B-type subunits into PP2A heterotrimers (B*AC), which block transformation. Specific targeting of PP2A B*AC complexes by MT and ST

oncoproteins promotes transformation. (B and C) HEK 293 cells were cotransfected with empty vector, HA-tagged wild-type PP2Ac (HA-PP2Ac wt), HA-tagged PP2Ac D85N mutant, HA-tagged PP2Ac carboxy-terminal leucine deletion mutant (HA-PP2Ac L309Δ), and SVST (B) or PyST (C) in the combinations indicated. HA-epitope tagged PP2Ac was immunoprecipitated 48h later with a sepharose bead-conjugated anti-HA antibody for 1.5h at 4°C with rocking to determine the binding of endogenous Bα and SVST (B) or PyST (C). After washing twice with PBS and lysis buffer, immune complexes (upper panels) and lysates (lower panels) were resolved on a 12% SDS-PAGE gel and probed with antibodies to the HA epitope tag, Bα subunit and SVST (B), or PyST (C). Figure and legend adjusted from [220].

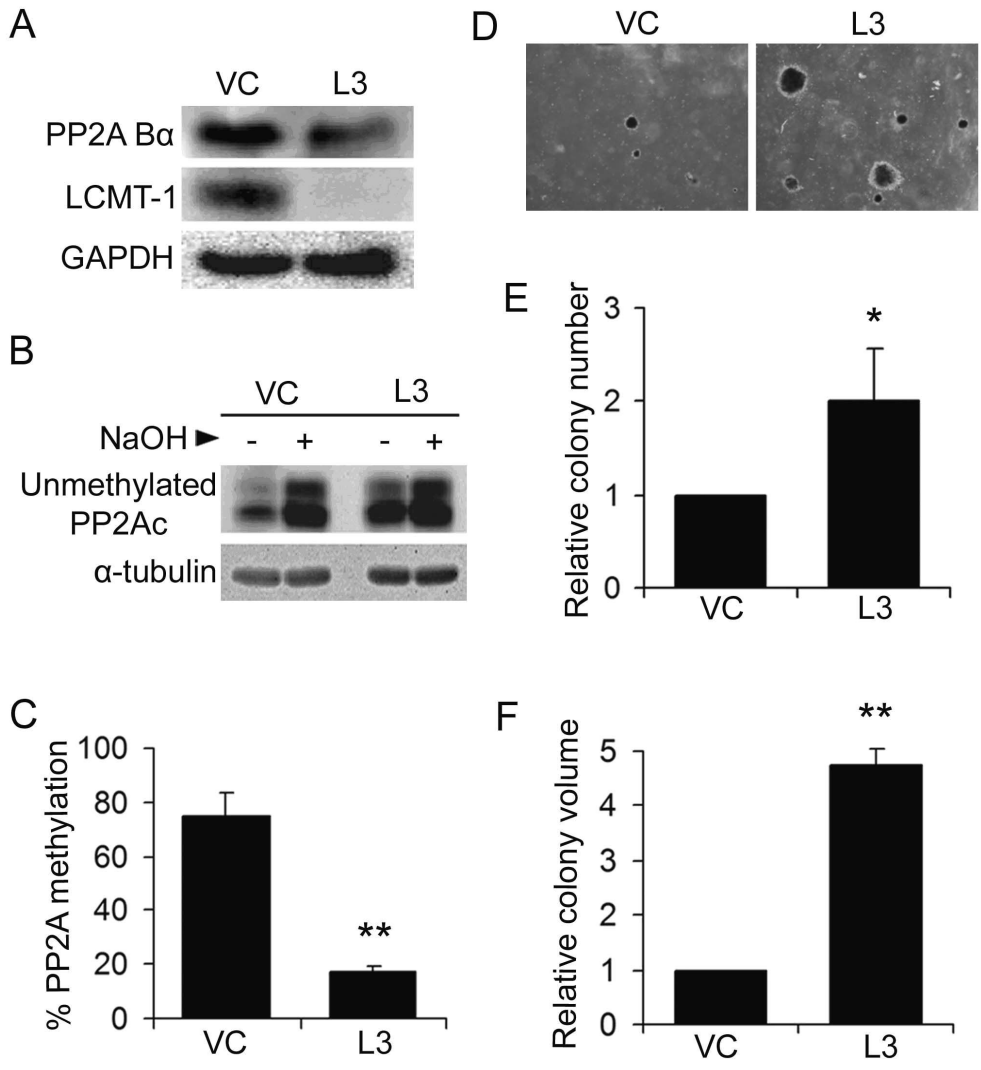


Figure 7. Knocking down LCMT-1 promotes transformation. (A) Knockdown of LCMT-1 in HEKTERASB56γ cells. HEKTERASB56γ cells stably expressing empty pLKO.1 vector control (VC) or LCMT-1 shRNA (L3) were lysed and LCMT-1 and Bα levels were detected by western blotting. GAPDH was used as loading control. (B and C) PP2Ac is highly unmethylated in the LCMT-1 knockdown line. (B) As described in Materials and Methods, equal volumes of lysates from VC and L3 cells were either treated with preneutralized base solution (- lanes; show unmethylated PP2Ac levels in cells) or base treated to completely demethylate PP2Ac and then neutralized (+ lanes;

100% demethylated controls) prior to being analyzed by western blotting for the level of unmethylated PP2Ac and α-tubulin (loading control). (C) The percent unmethylated PP2Ac was determined by quantitatively comparing the unmethylated PP2Ac signals in the – and + lanes. Percent methylated PP2Ac was calculated by subtracting percent unmethylated PP2Ac from 100. Graph depicts the average percent methylation of PP2Ac in VC and L3 knockdown lines. Error bars represent standard deviation (S.D.) of three independent experiments. (D) Anchorage-independent growth of VC and L3 knockdown cells in soft agar. Photographs show small, single, representative fields within the agar wells. Average colony numbers (E) and average colony volumes (F) were determined as described in Materials and Methods and data is shown in graphs as fold change relative to VC. Error bars represent S.D. of three independent experiments performed in triplicate. *, $P \leq 0.05$; ** $P \leq 0.01$. Figure and legend adjusted from [220].

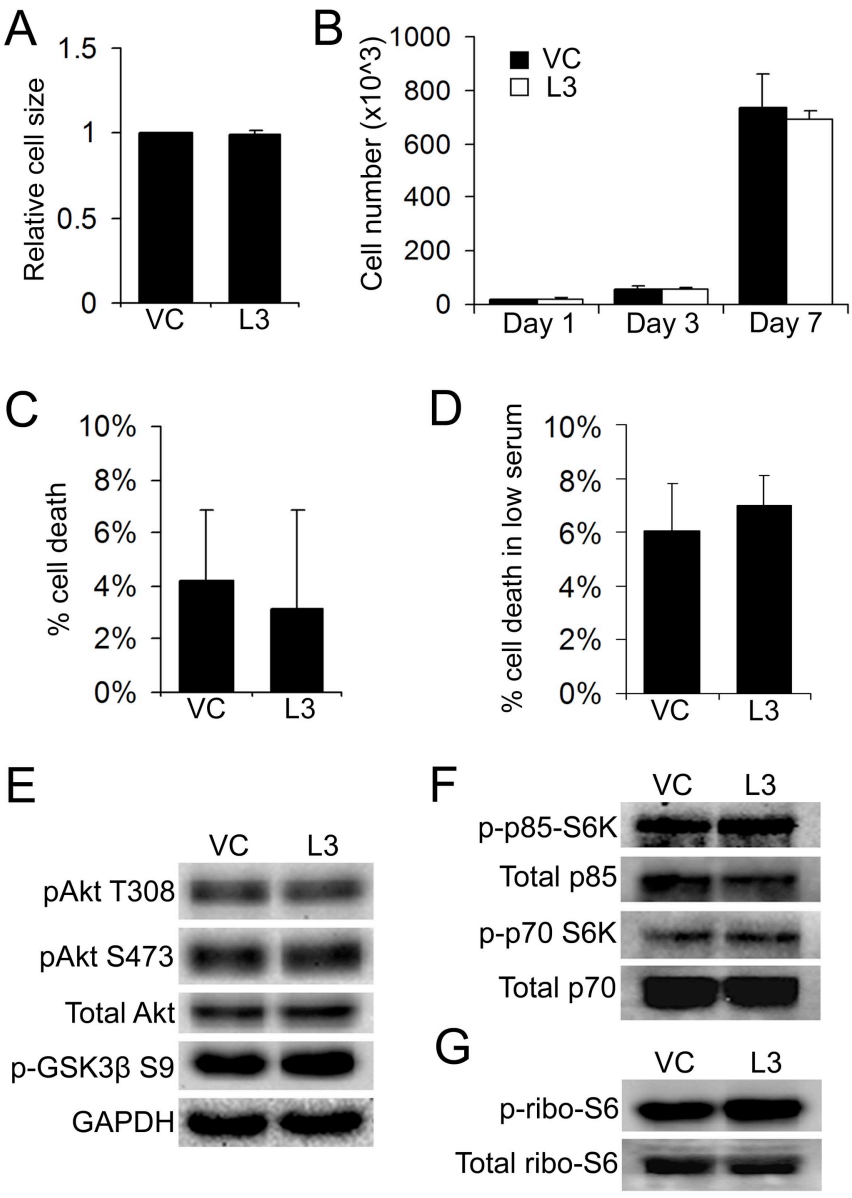


Figure 8. Adherent control and LCMT-1 knockdown lines show no difference in growth, death, or Akt and S6K signaling. (A) Non-confluent vector control (VC) and LCMT-1 knockdown (L3) cells growing on 10 cm tissue culture dishes for 48 hours were trypsinized, washed, and dispersed into single cell suspensions. $2x10^4$ cells/sample were analyzed using an AccuriC6 Flow Cytometer to compare cell size as determined by forward scatter-area (FSC-A). The average cell size is presented in the graph as fold

change relative to VC. (B) Growth rates of adherent VC and L3 knockdown lines were assessed by plating $1x10^4$ cells on 10 cm dishes on day 0 and collecting and counting all viable cells (adherent and detached) at days 1, 3 and 7. Cells were stained with trypan blue to exclude dead cells and counted by hemacytometer. Graph represents the average number of live cells at each time point. (C) The VC and L3 knockdown lines were plated at $5x10^4$ cells per 3.5 cm dish in triplicate and grown for 72 hours. Both adherent and detached cells were collected, stained with trypan blue to identify dead cells and counted by hemacytometer. The average percentage of dead cells of the VC and L3 knockdown lines is presented in the graph as % cell death. (D) VC and L3 cell lines were plated at $5x10^4$ cells per 3.5 cm dish in triplicate and cultured in DMEM supplemented with 10% FBS for 24 hours. Cells were then serum starved (0.1% FBS) for 48 hours and the percentage of dead cells was determined. The average percentage of dead cells in each line is presented in the graph. (E-G) Lysates from adherent VC and L3 knockdown cells were analyzed by western blotting for pAkt T308, pAkt S473, pGSK3β S9, p-p85 S6K, p-p70 S6K and p-rpS6. GAPDH, total Akt, total p70 and p85 S6K, and total rpS6 were used as loading controls. No statistically significant changes were seen in signaling in three independent experiments. Error bars in all panels represent S.D. of three experiments. *, $P \leq 0.05$; **, $P \leq 0.01$. Figure and legend adjusted from [220].

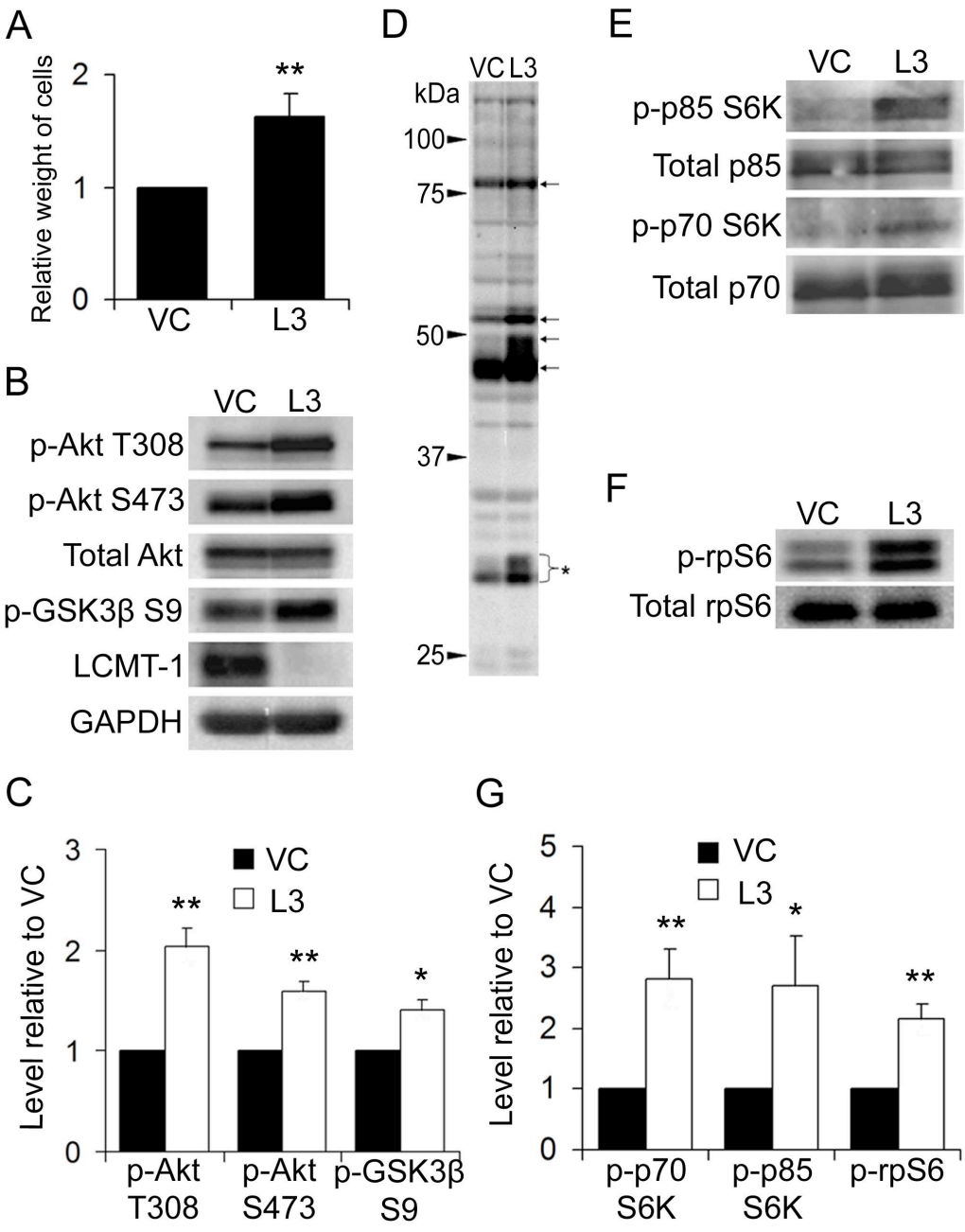


Figure 9. LCMT-1 knockdown activates Akt and S6K signaling in anchorage-independent conditions. (A) Equal numbers of vector control (VC) and LCMT-1 knockdown (L3) cells were seeded on low-binding tissue culture dishes to analyze differences during anchorage-independent growth. After 1 week, suspension cells were

weighed to assess growth. In the graph, data is presented as average fold change relative to VC for three independent experiments. (B) Lysates from VC and L3 knockdown suspension cells were analyzed by western blotting for changes in activation of Akt. GAPDH and total Akt were used as loading controls. Western blotting for LCMT-1 confirmed the knockdown of LCMT-1 in suspension cultures. (C) Graph depicts the average levels of phospho-Akt T308, phospho-Akt S473 and phospho-GSK3β S9 in three independent immunoblot experiments as fold change relative to VC. (D) An anti-Akt phospho-substrate motif (R/K)X(R/K)XX(pT/pS) antibody was used to probe the VC and L3 knockdown suspension cell lysates. Arrows highlight some proteins with increased phosphorylation in the L3 knockdown cells relative to VC. Bracket with asterisk indicates phospho-rpS6, which is known to cross-react with this antibody. (E and F) Lysates were probed for changes in p70 and p85 S6K activation and rpS6 phosphorylation. Total p70 and p85 S6K and total rpS6 were used as loading controls. (G) Graph represents average fold change in the levels of phospho-p85, phospho-p70 and phospho-rpS6 in three independent immunoblot experiments relative to VC. Experiments were performed in triplicate and error bars in all panels represent S.D. *, $P \leq 0.05$; **, $P \leq 0.01$. Figure and legend adjusted from [220].

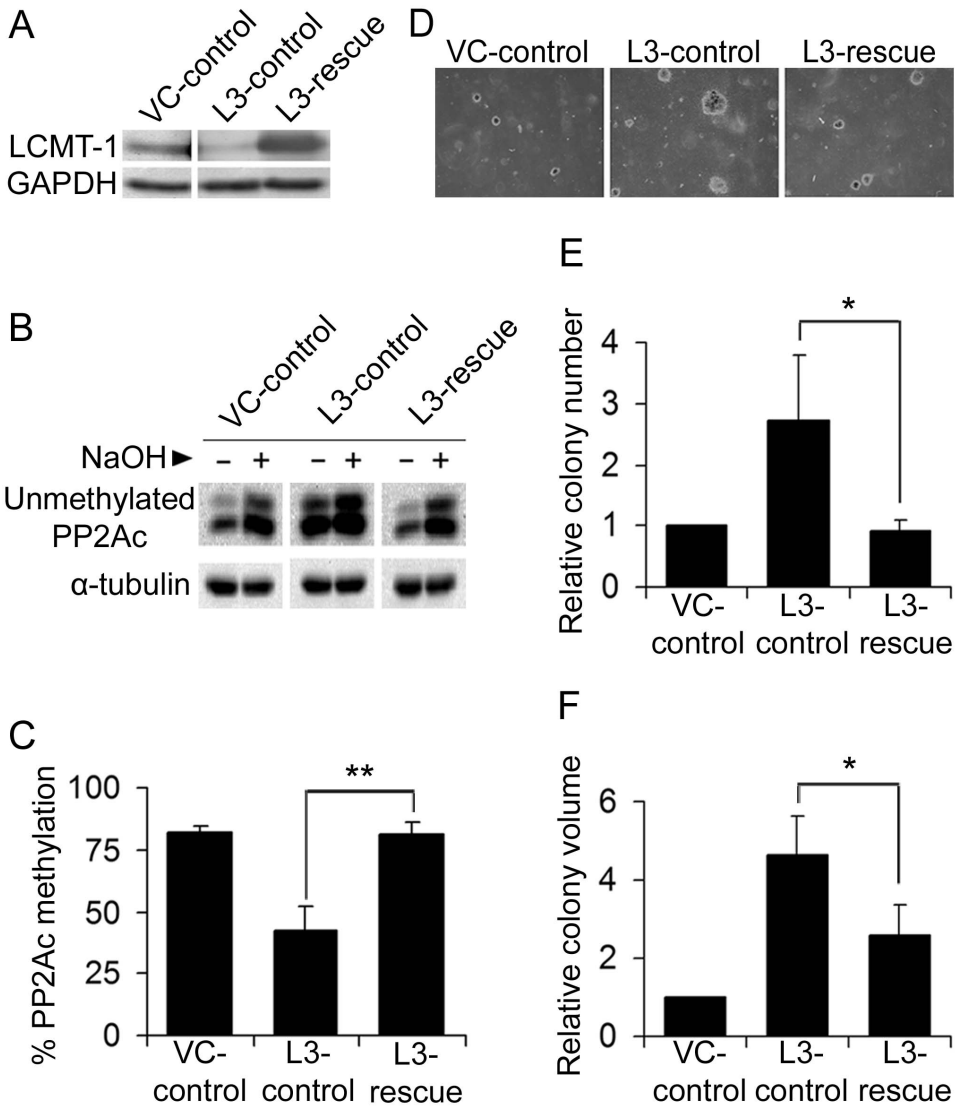


Figure 10. Effects of LCMT-1 knockdown on transformation are rescued by expression of an shRNA-resistant LCMT-1. (A) Rescue of the LCMT-1 knockdown line. Lysates from vector control (VC) and LCMT-1 knockdown (L3) cells stably expressing an empty control plasmid (VC-control and L3-control, respectively) and LCMT-1 knockdown cells stably expressing an LCMT-1 rescue plasmid (L3-rescue) were analyzed by western blotting for LCMT-1 protein expression. (B and C) PP2Ac methylation is rescued in the L3 rescue cell line. (B) Equal volumes of lysates from VC-

control, L3-control and L3-rescue were either treated with preneutralized base solution (- lanes; show unmethylated PP2Ac levels in cells) or base treated and then neutralized (+ lanes; 100% demethylated controls) prior to being analyzed by western blotting for the level of unmethylated PP2Ac and α-tubulin (loading control). (C) Graph depicts the average percent methylation of PP2Ac in the lysates, calculated as described in the legend to Fig. 6C. (D) Anchorage-independent growth of VC-control, L3-control, and L3-rescue cells in soft agar. Photographs show small, single, representative fields within the agars. Average colony numbers (E) and average colony volumes (F) were determined and data is shown in graphs as fold change relative to VC-control. Error bars in all panels represent S.D. of three independent experiments. *, $P \leq 0.05$; ** $P \leq 0.01$. Figure and legend adjusted from [220].

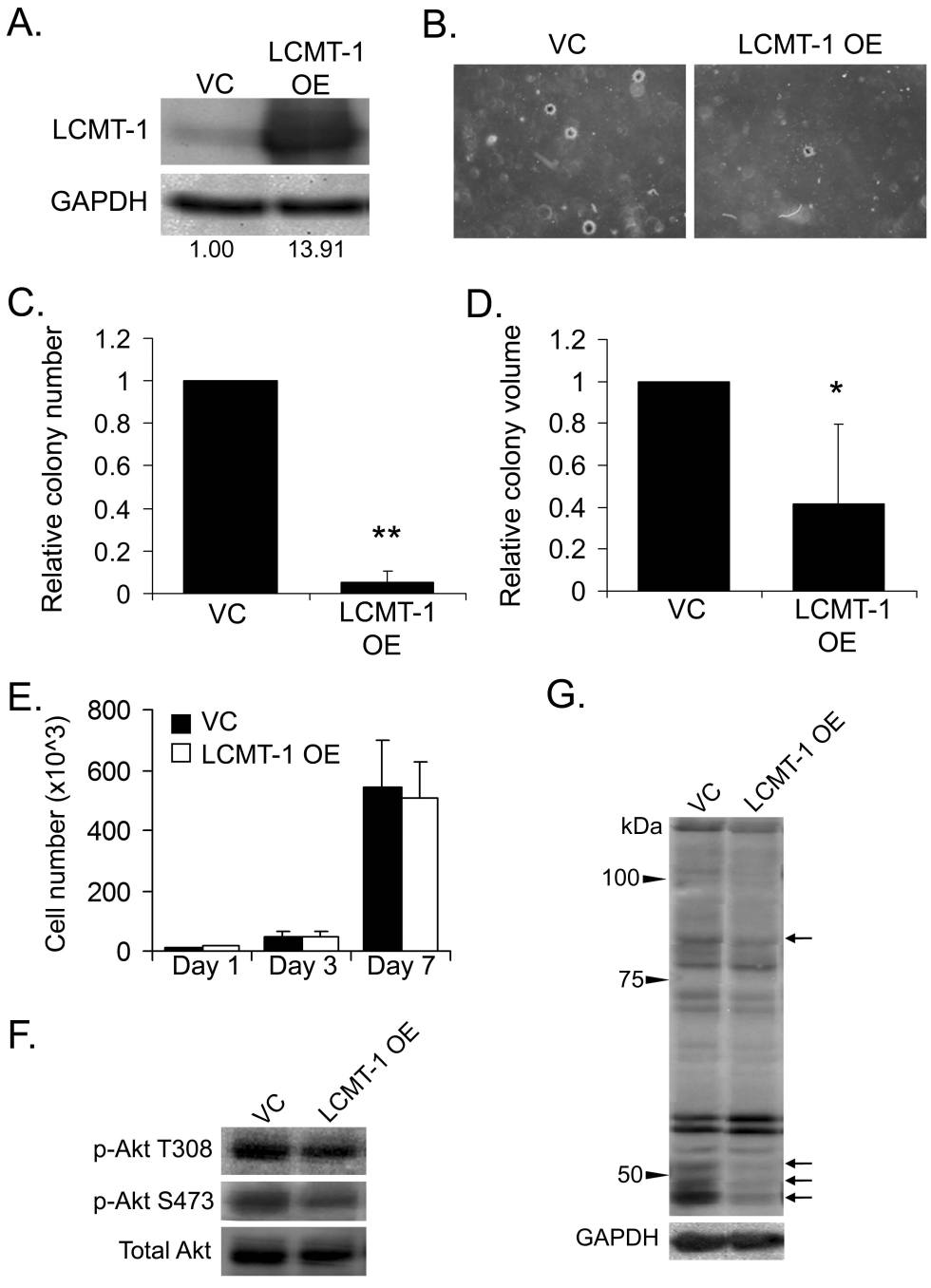


Figure 11. LCMT-1 overexpression confirms the tumor suppressive role of PP2Ac methylation. (A) Lysates from HEKTERASB56γ cells stably expressing either an empty vector (VC) or excess LCMT-1 (LCMT-1 OE) were analyzed by western blotting for the

expression of LCMT-1. (B) Anchorage-independent growth of VC and LCMT-1 OE cells in soft agar. Photographs show small, single, representative fields within the agars. Average colony numbers (C) and average colony volumes (D) were determined and data are shown in graphs as fold change relative to VC. Error bars represent S.D. of three independent experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq 0.01$. (E) Growth rates of adherent VC and LCMT-1 OE cells were assessed as described in 3B. Graph represents the average number of live cells at Days 1, 3, and 7. (F) Lysates from VC and LCMT-1 OE suspension cells were analyzed by western blotting for changes in activation of Akt. Total Akt was used as loading control. (G) Akt phospho-substrate antibody was used to probe the VC and LCMT-1 OE lysates. Arrows highlight some proteins whose phosphorylation increased in LCMT-1 OE cells.

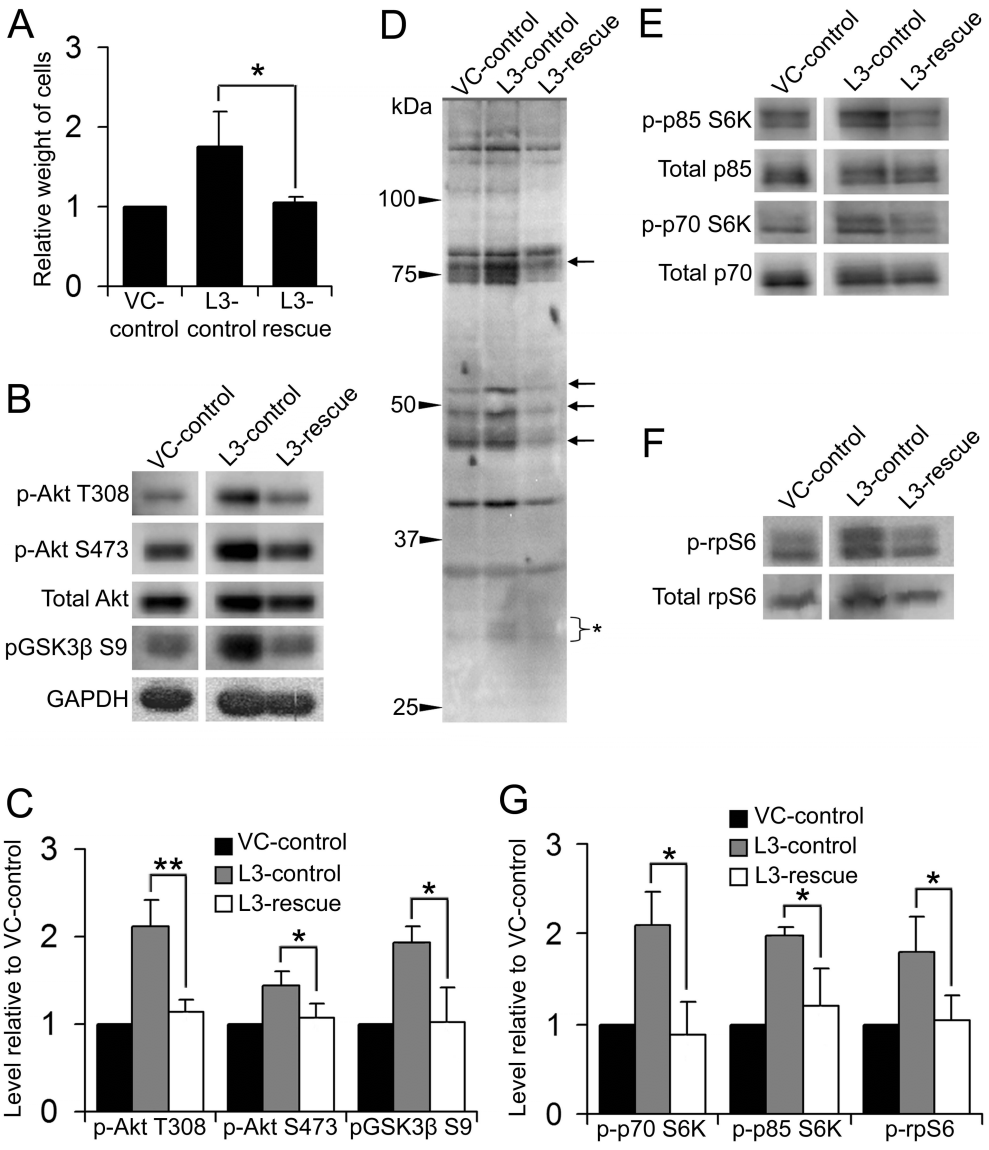


Figure 12. Effects of LCMT-1 knockdown on biochemical signaling are rescued by re-expression of LCMT-1. (A) Normalization of anchorage-independent growth by LCMT-1 re-expression. Anchorage-independent growth of VC-control, L3-control and L3-rescue cells (defined in Fig. 5A) were assessed as in Fig. 4A by weighing cells after 1 week in suspension culture. Data is presented as fold-change relative to VC. (B, C, and D) Normalization of Akt signaling by LCMT-1 re-expression. (B) Lysates from VC-

control, L3-control, and L3-rescue suspension cells were analyzed by western blotting for changes in activation of Akt. GAPDH and total Akt were used as loading controls. (C) Graph depicts the average fold change in the levels of phospho-Akt T308, phospho-Akt S473 and phospho-GSK3β S9 in three immunoblot experiments relative to VC-control. (D) Akt phospho-substrate antibody was used to probe lysates from the VC-control, L3-control, and L3-rescue lines. Arrows indicate some proteins whose phosphorylation increased upon L3 knockdown but returned to control levels upon re-expression of LCMT-1. Bracket indicates phospho-rpS6. (E and F) Rescue of S6K activation and rpS6 phosphorylation by LCMT-1 re-expression. Lysates were probed for changes in the activation state of p70 and p85 S6K and rpS6 phosphorylation. Total p70 and p85 S6K and total rpS6 were used as loading controls. (G) Graph represents average fold change in the levels of phospho-p85, phospho-p70 and phospho-rpS6 in three immunoblot experiments relative to VC-control. Error bars in all panels represent S.D. of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$. Figure and legend adjusted from [220].

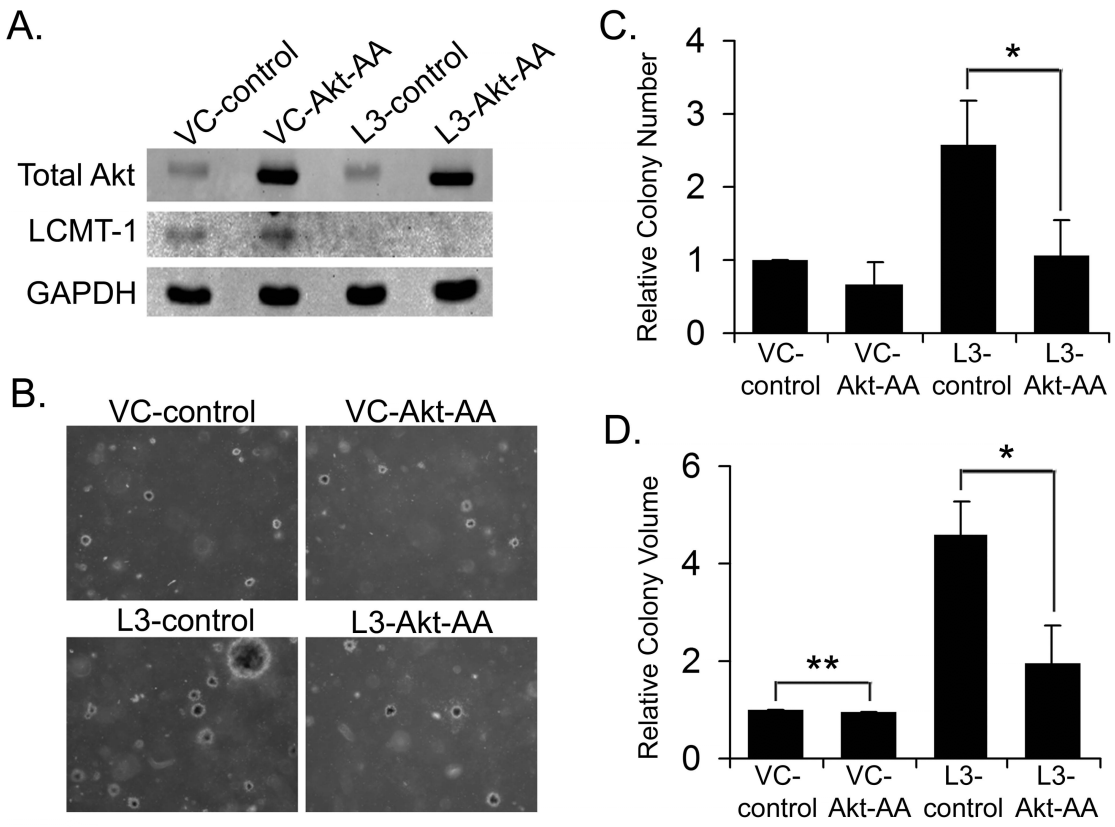


Figure 13. Akt activation is necessary for the enhanced transformation caused by LCMT-1 knockdown. (A) Lysates from vector control (VC) and LCMT-1 knockdown (L3) cells stably expressing an empty control plasmid (VC-control and L3-control, respectively) and lysates from VC and L3 knockdown cells stably expressing dominant negative Akt-AA (VC-Akt-AA and L3-Akt-AA, respectively) were analyzed by western blotting for the expression of Akt-AA, LCMT-1, and GAPDH. (B) Anchorage-independent growth of VC-control, VC-Akt-AA, L3-control and L3-Akt-AA cells in soft agar. Photographs show small, single, representative fields within the agars. Average colony numbers (C) and average colony volumes (D) were determined and data is shown in graphs as fold change relative to VC-control. Error bars represent S.D. of three independent experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq 0.01$.

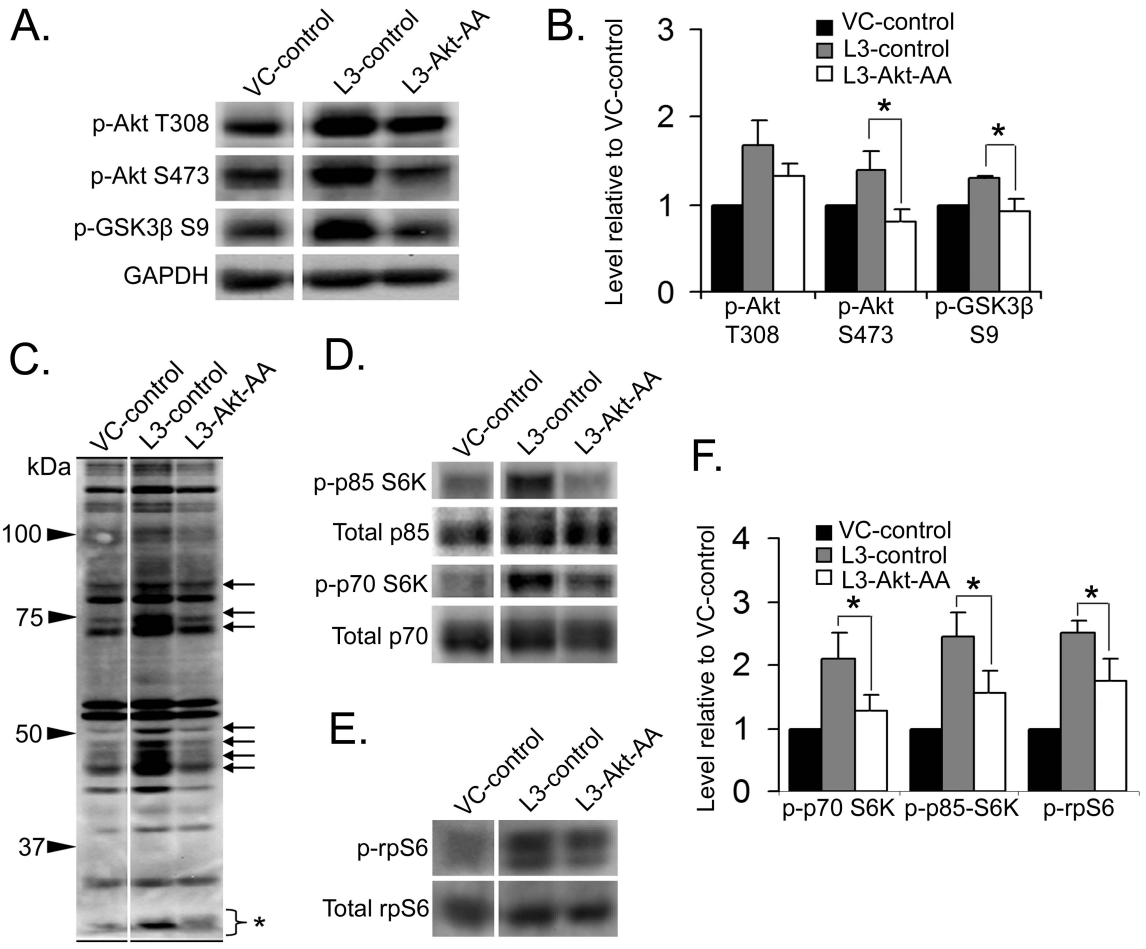


Figure 14. Effects of LCMT-1 knockdown on biochemical signaling are reversed by expression of dominant-negative Akt. (A) Lysates from VC-control, L3-control and L3-Akt-AA suspension colonies were analyzed by western blotting for changes in activation of endogenous Akt. GAPDH was used as a loading control. (B) Graph depicts the average fold change in the levels of phospho-Akt T308, phospho-Akt S473, and phospho-GSK3β S9 in three immunoblot experiments relative to VC-control. (C) Probing of VC-control, L3-control, and L3-Akt-AA lysates with phospho-Akt substrate antibody shows that dominant negative Akt expression prevents the increased phosphorylation of many proteins caused by LCMT-1 knockdown (arrows show examples). The bracket with

asterisk indicates phospho-rpS6, which ran at the bottom of this 7.5% SDS-PAGE gel. (D and E) Lysates were probed for changes in p70 and p85 S6K activation and rpS6 phosphorylation. Total p70 and p85 S6K and total rpS6 were used as controls. (F) Graph depicts the average fold change in the levels of phospho-p85, phospho-p70 and phospho-rpS6 in three immunoblot experiments relative to VC-control. Error bars represent S.D. of three independent experiments. For all graphs: *, $P \leq 0.05$; **, $P \leq 0.01$. Figure and legend adjusted from [220].

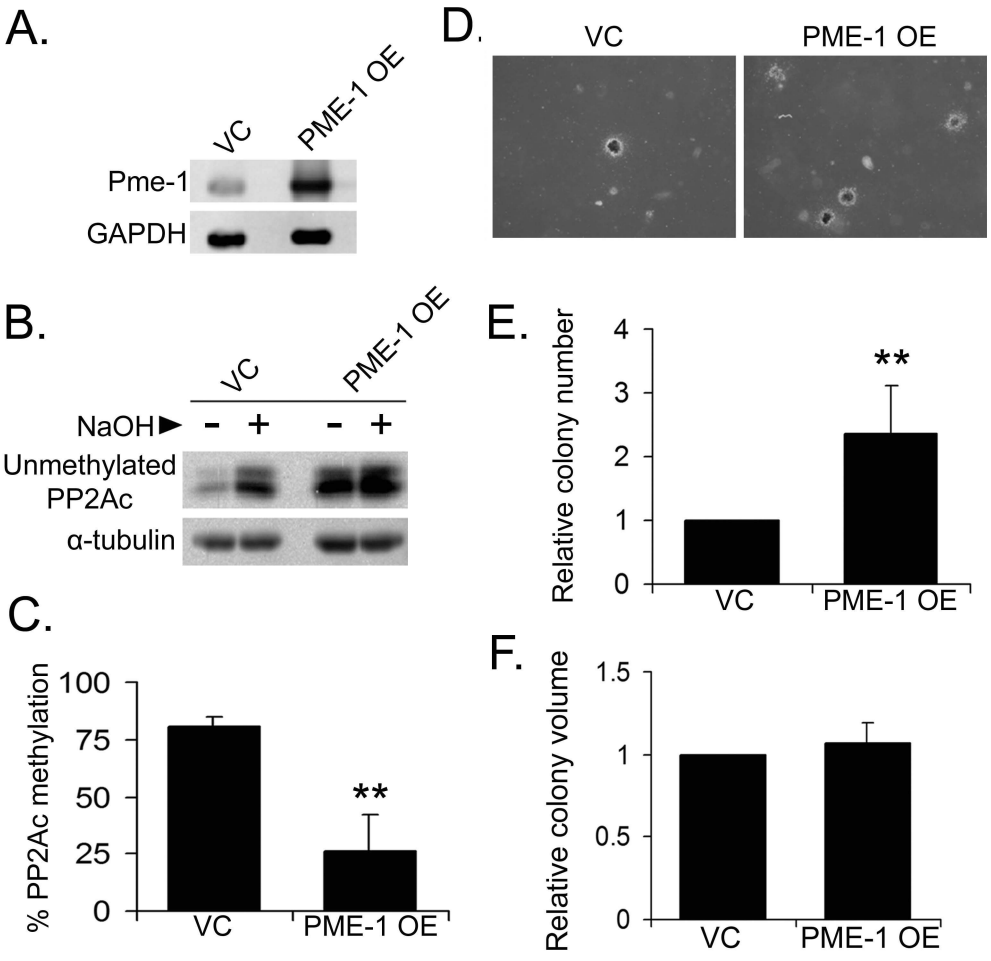


Figure 15. Overexpression of the PP2A methylesterase, PME-1, enhances transformation. (A) Lysates from HEKTERASB56γ cells stably expressing either an empty vector (VC) or excess PME-1 (PME-1 OE) were analyzed by western blotting for the expression of PME-1. (B and C) PP2Ac methylation is reduced in the PME-1 OE cell line. (B) Equal volumes of lysates from VC and PME-1 OE lines were either treated with preneutralized base solution (- lanes; show unmethylated PP2Ac levels in cells) or base treated and then neutralized (+ lanes; 100% demethylated controls) prior to being analyzed by western blotting for the level of unmethylated PP2Ac and α-tubulin (loading control). (C) Graph depicts the average percent methylation of PP2Ac in the lysates,

calculated as described in the legend to Fig. 6C. Error bars represent S.D. of three independent experiments. (D) Anchorage-independent growth of VC and PME-1 OE cells in soft agar. Photographs show small, single, representative fields within the agars. Average colony numbers (E) and average colony volumes (F) were determined and data is shown in graphs as fold change relative to VC. Error bars represent S.D. of six independent experiments performed in triplicate. **, $P \leq 0.01$. Figure and legend adjusted from [220].

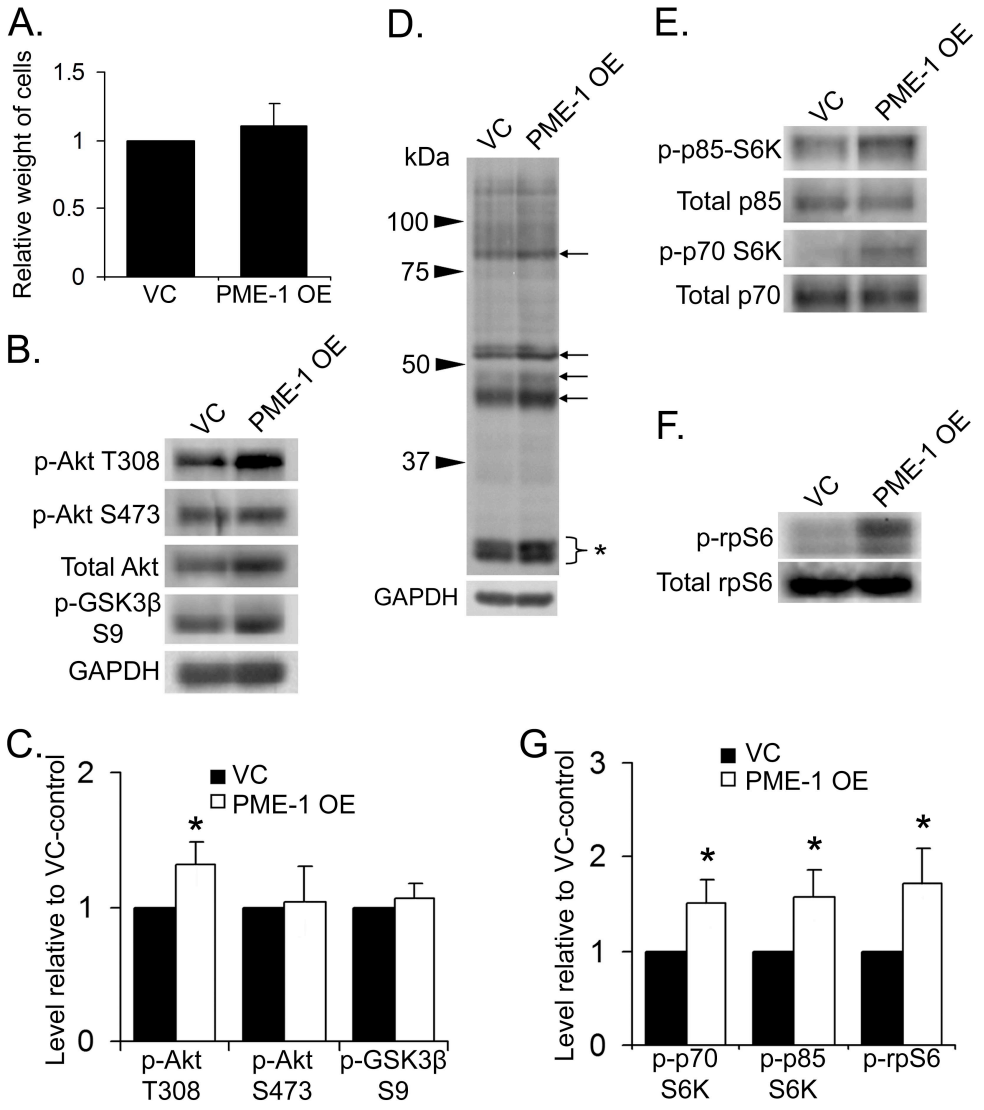


Figure 16. Overexpression of the PP2A methylesterase, PME-1, elicits similar biochemical changes as LCMT-1 suppression. (A) Anchorage-independent growth of VC and PME-1 OE cells were assessed as in Fig. 4A by weighing cells after 1 week in suspension culture. Data is presented as fold-change relative to VC (B) Lysates from VC and PME-1 OE suspension cells were analyzed by western blotting for changes in activation of Akt. GAPDH was used as a loading control. (C) Graph depicts the average fold change in the levels of phospho-Akt T308, phospho-Akt S473 and phospho-GSK3β

S9 in three independent immunoblot experiments relative to VC. (D) Akt phospho-substrate antibody was used to probe the VC and PME-1 OE lysates. Arrows highlight some proteins whose phosphorylation increased in PME-1 OE cells. Bracket with asterisk indicates phospho-rpS6. (E and F) Lysates were probed for changes in p70 and p85 S6K activation and rpS6 phosphorylation. Total p70 and p85 S6K and total rpS6 were used as controls. (G) Graph represents the average fold change in the levels of phospho-p85, phospho-p70 and phospho-rpS6 in three immunoblot experiments relative to VC. Error bars represent S.D. of three independent experiments. For all graphs: *, $P \leq 0.05$. Figure and legend adjusted from [220].

CHAPTER 3.2

Results

Investigating the Potential Targets of SV40 Small T Antigen and the Potential Contributions of the SV40 Small T Antigen PP2A-independent Region to Transformation

Hypothesis: The PP2A-independent region of SV40 small T (SVST) or inhibition of methylation-sensitive, T-antigen targeted PP2A B-type subunits may be important for SVST-mediated transformation.

Predictions: Expressing the PP2A-independent region of SVST or suppressing PP2A B-type subunits targeted by SVST, in combination with B'γ knockdown, will better recapitulate SVST's robust transformative capacity.

Expression of the PP2A-independent region of SVST does not complement B'γ knockdown to further enhance transformation

The N-terminal, PP2A-independent region of SVST is not sufficient to promote transformation in HEKTER cells [178] and is not itself required for interaction of SVST and PP2A; however it does enhance SVST's affinity for PP2A [179]. Furthermore, this region participates in chaperone activity and cell cycle regulation [180, 181], which may contribute to the transformative properties of SVST in conjunction with suppression of

PP2A complexes. Therefore, I hypothesized that expressing the first 110 amino acids of SVST (ST110), the PP2A-independent region of SVST (Figure 17A), may complement the B'γ knockdown in the HEKTERASB56γ cell line to further support cellular transformation. To test this hypothesis, HEKTERASB56γ cells were infected with vector control (VC) or ST110-expressing lentivirus and Western blot analysis was used to verify the stable expression of ST110 (Figure 17B). To examine the contribution of the PP2A-independent region of SVST to transformation, VC and ST110 cells were evaluated for anchorage-independent growth in soft agar. After 3 weeks of growth in agar, colonies were photographed (Figure 17C) and examined by microscopy for differences in colony number and size. Surprisingly, expression of ST110 did not complement the B'γ knockdown to enhance transformation in HEKTERASB56γ cells. Rather, expression of ST110 significantly reduced colony number by 76% (Figure 17D) and colony volume by over 30% (Figure 17E). Therefore, ST110 could not contribute to transformation in this system and instead may exert a dominant-negative effect on the transformed phenotype of the HEKTERASB56γ cell line. In other words, the PP2A-independent region of SVST may interact with one or more unknown, antiproliferative, antisurvival targets thereby counteracting the progrowth, prosurvival signaling provided by the B'γ knockdown.

Suppression of methylation-sensitive, SVST-targeted PP2A B-type subunits in combination with B'γ knockdown reveals differential effects on cell growth, survival, and transformation

SVST, like PyST and PyMT, blocks PP2A function by replacing certain B-type subunits resulting in transformation. Specifically, Bα, B'α, and B'γ, have been identified

as being targeted for replacement by MT and/or ST oncoproteins [98, 171, 173, 175]. Interestingly, of the three cellular B-type subunits reported to be displaced by the MT or ST oncoproteins, two (Bα and B'α) are methylation-sensitive and one (B'γ) is likely to be methylation-sensitive [43, 44, 46, 52, 216]. Furthermore, based on the high sequence homology between Bα and Bδ, it likely that Bδ is also methylation-sensitive and targeted by MT and ST oncoproteins. In Chapter 3.1, I investigated the effects of inhibiting PP2Ac methylation by targeting the PP2Ac methyltransferase, LCMT-1, and determined that suppression of PP2Ac methylation enhanced cellular transformation. In order to understand the individual and distinct contributions of each methylation-sensitive B-type subunit, I knocked down Bα, and B'α individually in the HEKTERASB56γ cell line to determine if either of these methylation-sensitive B-type subunits could enhance anchorage-independent growth in soft agar and better recapitulate the robust transformation seen with SVST expression in HEKTER cells. Furthermore, I knocked down Bδ in HeLa cells and observed parallel effects between Bδ suppression and SVST expression on mitosis.

Suppressing Bα. To explore the effect of knocking down PP2A-Bα complexes, HEKTERASB56γ cells were infected with vector control (VC) or Bα-directed (B3) shRNA virus, and Western blot analysis was used to verify the knockdown of Bα (Figure 18A). Quantitation of three independent experiments showed that the Bα shRNA stably reduced Bα expression by 86% ± 5% compared with the VC cells. In addition, the steady-state expression level of Bα was also reduced in LCMT-1 knockdown line showing that the Bα subunit is methylation-sensitive. To examine the role of Bα in transformation, VC and B3 cells were evaluated for anchorage-independent growth in soft agar. After 3

weeks of growth in agar, colonies were examined by microscopy and quantitative analysis showed that B3 cells had a two-fold, albeit not statistically significant, increase in colony number (Figure 18B) and showed no change in colony volume compared to VC cells (Figure 18C). These results indicate that while targeting Bα may contribute slightly to the cumulative effects of SVST-mediated transformation, it cannot on its own promote robust transformation. To further understand why Bα did not significantly enhance transformation as predicted by the hypothesis, B3 cells were analyzed for differences in growth and survival compared to VC cells. Comparison of the proliferation rates of VC and B3 cells over a 4-day period showed no significant differences between the two cell lines, indicating that Bα knockdown does not alter cell proliferation in anchorage-dependent conditions (Figure 18D). Quantitation of the amount of cell death in VC and B3 cultures during adherent cell growth in normal media with serum showed that Bα knockdown cells had increased cell death but the increase was not statistically significant (Figure 18E). Therefore, VC and B3 cells were grown in low serum conditions (0.1% FBS) for 48 hours to stress the cells, which did reveal an effect on survival. B3 cells exhibited more sensitivity to low serum conditions with over a two fold increase in cell death compared to VC cells (Figure 18F). This result suggests that Bα is important for survival signaling in low nutrient conditions providing a possible explanation for the modest growth in soft agar.

Suppressing B'α. To explore the effect of knocking down PP2A-B'α complexes, HEKTERASB56γ cells were infected with vector control (VC) or B'α-directed (H6) shRNA virus, and Western blot analysis was used to verify the knockdown of B'α (Figure 19A). Quantitation of the Western blot showed that the B'α shRNA stably

reduced B'α expression 60% compared with the VC cells. To examine the role of B'α in transformation, VC and H6 cells were evaluated for anchorage-independent growth in soft agar. After 3 weeks of growth in agar, colonies were examined by microscopy and quantitative analysis showed that H6 cells had a statistically significant 69% and 32% reduction in colony number and volume, respectively (Figures 19B and C). Additionally, a second B'α-targeting shRNA (H5) was used to confirm the reduction in transformation and soft agar analysis of cells knocked down for B'α by the H5 shRNA revealed statistically significant reductions in colony number by 96% and colony volume by 37% (Figure 19B and C). These results were particularly surprising on first glance as they did not seem to fit with the hypothesis that targeting methylation-sensitive PP2A complexes would promote transformation as seen with the direct suppression of PP2Ac by LCMT-1 knockdown. Instead, suppression of B'α reduced transformation in HEKTERASB56γ cells, and thus does not complement B'γ knockdown to recapitulate SVST-mediated transformation. Nonetheless, this result was quite interesting and presents the possibility that suppressing B'α-containing complexes may be useful as an anti-cancer target. To further divulge how targeting B'α reduces anchorage-independent growth, I tested whether the decrease in colony growth of B'α knockdown cells in soft agar reflects changes in proliferation and/or death rates in standard, anchorage-dependent tissue culture. Comparison of the proliferation rates of VC and H6 cells over a 4-day period and of cell death rates in normal and low serum conditions revealed no significant differences between the two lines in anchorage-dependent conditions (Figures 19D-F). Therefore, suppression of B'α reduces cellular growth and/or survival specifically in anchorage-independent conditions, and thus may have potential as an anti-cancer strategy.

Suppressing Bδ. During the initial stages of selecting the most potent shRNAs for knockdown in HEKTERASB56γ cells, HeLa cells are used to titer shRNA-expressing viruses to determine the concentration of viral particles per milliliter of virus produced. Furthermore, infected HeLa cells are then subjected to western analysis to assess the knockdown efficiency for each shRNA, thereby revealing the best viral candidate for infection of HEKTERASB56γ cells. Due to the lack of availability of a Bδ antibody, HeLa cells infected with VC or Bδ-directed shRNA viruses (B10-B12) were transfected with a HA-tagged PP2A Bδ and also cotransfected with GFP as a readout of transfection efficiency. Forty-eight hours post-transfection, HeLa cells were lysed and analyzed by Western blotting for the level of Bδ knockdown. Using GFP as a total transfection readout and PP2Ac as a loading control, Bδ was found to be reduced in the B10, B11, and B12 knockdown lines by 73%, 21% and 40%, respectively when compared to VC (Figure 20A). Thus, the B10 and B12 Bδ-targeting lentiviruses were deemed the most potent and were, therefore, utilized for further experimentation. Interestingly, after several days in culture, HeLa cells infected with the B10 and B12 Bδ-targeting lentiviruses became visibly distressed and would undergo apoptosis. This preliminary finding became of great interest considering the fact that Bδ is involved in cell cycle regulation. Therefore, it seemed likely that the knockdown was contributing to mitotic dysfunction. Of note, expression of SVST has also been linked to the deregulation of the cell cycle and thus it is feasible that it does so by targeting Bδ-containing PP2A complexes. To further explore these speculations, HeLa cells were utilized to assess mitotic defects associated with Bδ knockdown or SVST expression.

To confirm and document what had been visually observed, HeLa cells were infected with VC, B10 and B12 lentiviruses at an MOI of 3 to ensure that >95% of cells would receive at least one viral particle. Forty-eight hours post-infection, infected cells were observed by phase-contrast, time-lapse microscopy over a 4-day period (Figure 20B). At the start of the time-lapse, HeLa VC, B10 and B12 cells appear to be very similar in cell morphology and colony size. After 24 hours (or 3 days post-infection), the control and knockdown cells still appear similar; however, the B10 and B12 knockdown cells have several apoptotic cells as observed by the "rounded-up" white structures within the colonies (arrows). At the 72 hour mark (or 5 days post-infection), the control cells are healthy and colony size is growing at a normal rate. In contrast, the knockdown cells are exhibiting erratic cell migration, in the case of B10, and an obvious stagnation of colony growth due to an increasing number of unhealthy and apoptotic cells in both knockdown lines (arrows). Lastly, after 96 hours (or 6 days post-infection), control cells again appear normal and are robustly proliferating whereas the knockdown lines are clearly showing signs of reduced growth and/or survival. These results indicate that suppressing Bδ is detrimental for cell cycle function and is likely affecting growth and survival through mitotic dysfunction.

To test this assumption, HeLa cells expressing H2B-GFP, which allows for the visualization of DNA (histones) and therefore mitotic events, were infected with VC, B10 and B12 lentivirus and subjected to time-lapse microscopy 24 hours post-infection (Figure 21A). At the start of the time-lapse, HeLa VC, B10 and B12 cells appear to be very similar and are growing and surviving. At 24 hours (or 2 days post-infection), control cells are dividing normally but the knockdown cells are beginning to exhibit

survival issues with a number of apoptotic remnants already visible as punctate objects with no fluorescent green DNA (arrows). At 48 hours (or 3 days post-infection), the differences between the control and knockdown cells becomes more obvious (arrows) and at 60 hours, control cells show a high amount of survival while the knockdown cells have relatively few surviving cells. Those cells that are still alive appear to exhibit major issues with both entry and progression of mitosis. At 60 hours, most Bδ knockdown cells do not enter mitosis and those that do, inevitably die. In Figure 21B a zoomed in view of a representative cell undergoing mitosis in the VC, B10, and B12 cells is provided to highlight the dysfunctional mitotic events occurring in Bδ knockdown cells. In control cells, commencement, progression and exiting of mitosis is normal and takes a little over an hour. In contrast, the B10 knockdown cells that attempt to undergo mitosis appear to have major issues with chromosome alignment at the metaphase plate and after several hours will transition to anaphase without proper alignment, resulting in apoptosis. In B12, cells that attempt mitosis also appear to have dysfunctional chromosomal alignment and rather than dividing, undergo apoptosis. These results highlight the importance of Bδ in regulating mitosis and provide new evidence that partially suppressing the pool of PP2A-Bδ-containing complexes can lead to cell cycle dysfunction.

As mentioned, SVST has also been linked to cell cycle regulation and because SVST likely replaces Bδ from PP2A complexes, it is probable that SVST expression will illicit similar mitotic defects in HeLa cells as Bδ knockdown. To test this prediction, HeLa-H2B-GFP expressing cells were cotransfected with either an empty control plasmid (VC) or a SVST expression plasmid (SVST) and a transfection readout fluorescent marker, mCherry. After 48 hours post-transfection, cells were subjected to time-lapse

microscopy for a 3-day period to observe mitotic events. HeLa-H2B-GFP control cells typically underwent normal mitosis whereas cells expressing SVST appeared to have mitotic issues at the same stage as Bδ knockdown cells. In Figure 21C, a representative cell undergoing mitosis in the VC and SVST expressing cells is provided. In VC cells, cells enter mitosis, transition from metaphase to anaphase and successfully exit mitosis in a little over an hour. The SVST expressing cells, on the other hand, exhibit major issues with aligning chromosomes at the metaphase plate, as can be visualized by lagging chromosomes, and after several hours, undergo apoptosis. This finding is highly reminiscent of the mitotic defects seen in Bδ knockdown cells and supports the claim that SVST targets Bδ and can deregulate the cell cycle likely through this connection. Although suppressing Bδ or expressing SVST in HeLa cells causes widespread apoptosis, the effect of suppressing Bδ in HEKTERASB56γ cells may still contribute to transformation based on the fact that expression of SVST in HEKTERASB56γ cells does not cause apoptosis but rather enhances transformation. This is likely due to the fact that HeLa cells have not acquired the same oncogenic pathway activations as the HEKTERASB56γ cells and, therefore, may not have the capability to overcome the pro-apoptotic signals activated by Bδ knockdown or SVST expression. Therefore, the effects of suppressing Bδ may promote transformation in the HEKTERASB56γ system; however, this remains to be determined.

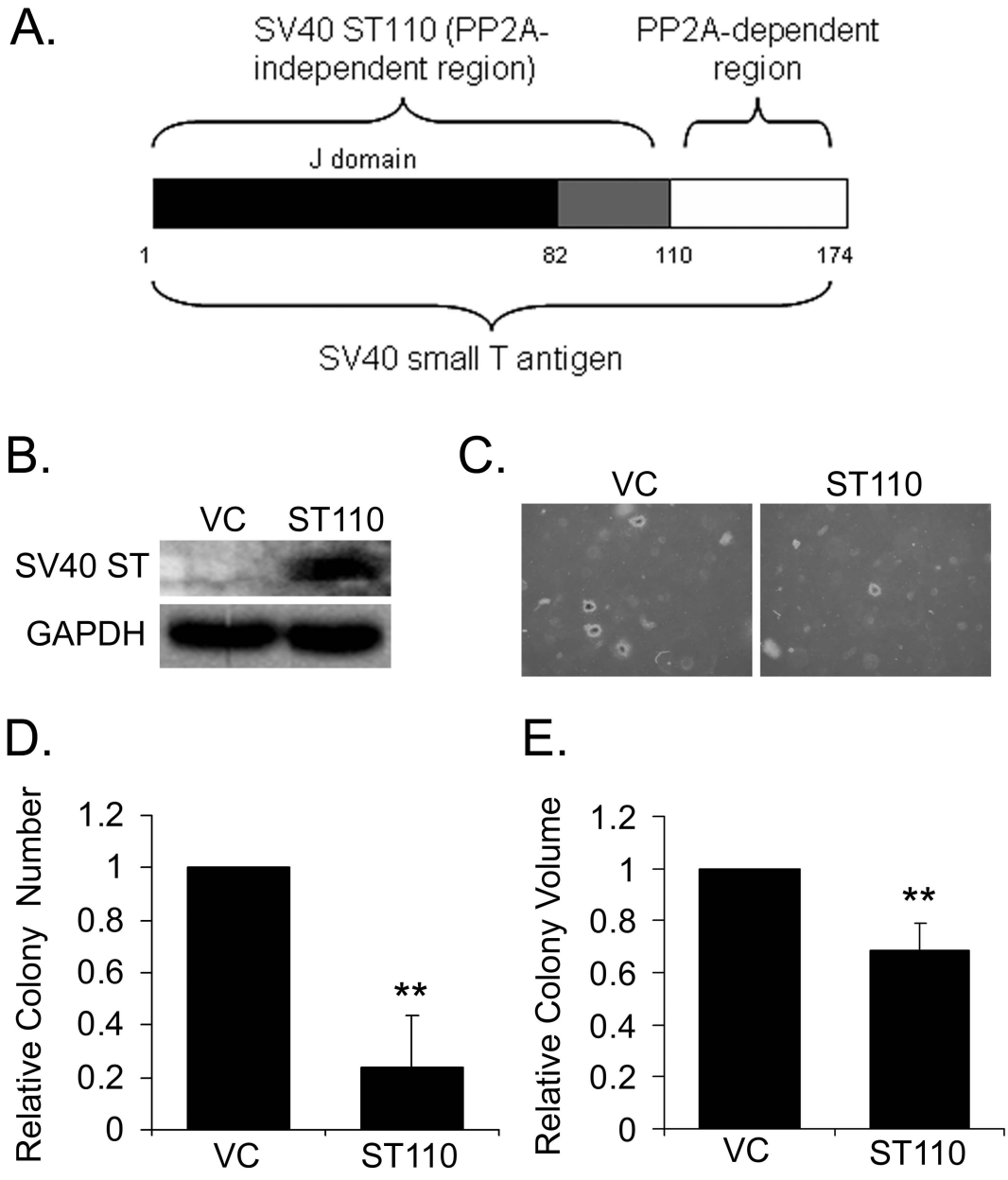


Figure 17. Expression of the PP2A-independent region of SVST does not complement B'γ knockdown to further enhance transformation. (A) The diagram indicates the distinct regions of SV40 small T antigen (SVST) which include the N-terminal, PP2A-independent region (amino acids 1-110) and the C-terminal, PP2A-dependent region (amino acids 110-174). (B) Expression of the PP2A-independent region

of SVST (ST110) in HEKTERASB56γ cells. HEKTERASB56γ cells stably expressing empty vector control (VC) or ST110 were lysed and ST110 levels were detected by western blotting. GAPDH was used as loading control. (C-E) Anchorage-independent growth of VC and ST110-expressing cells in soft agar. (C) Photographs show small, single, representative fields within the agar wells. Average colony numbers (D) and average colony volumes (E) were determined and data is shown in graphs as fold change relative to VC. Error bars represent S.D. of three independent experiments performed in triplicate. ** $P \leq 0.01$.

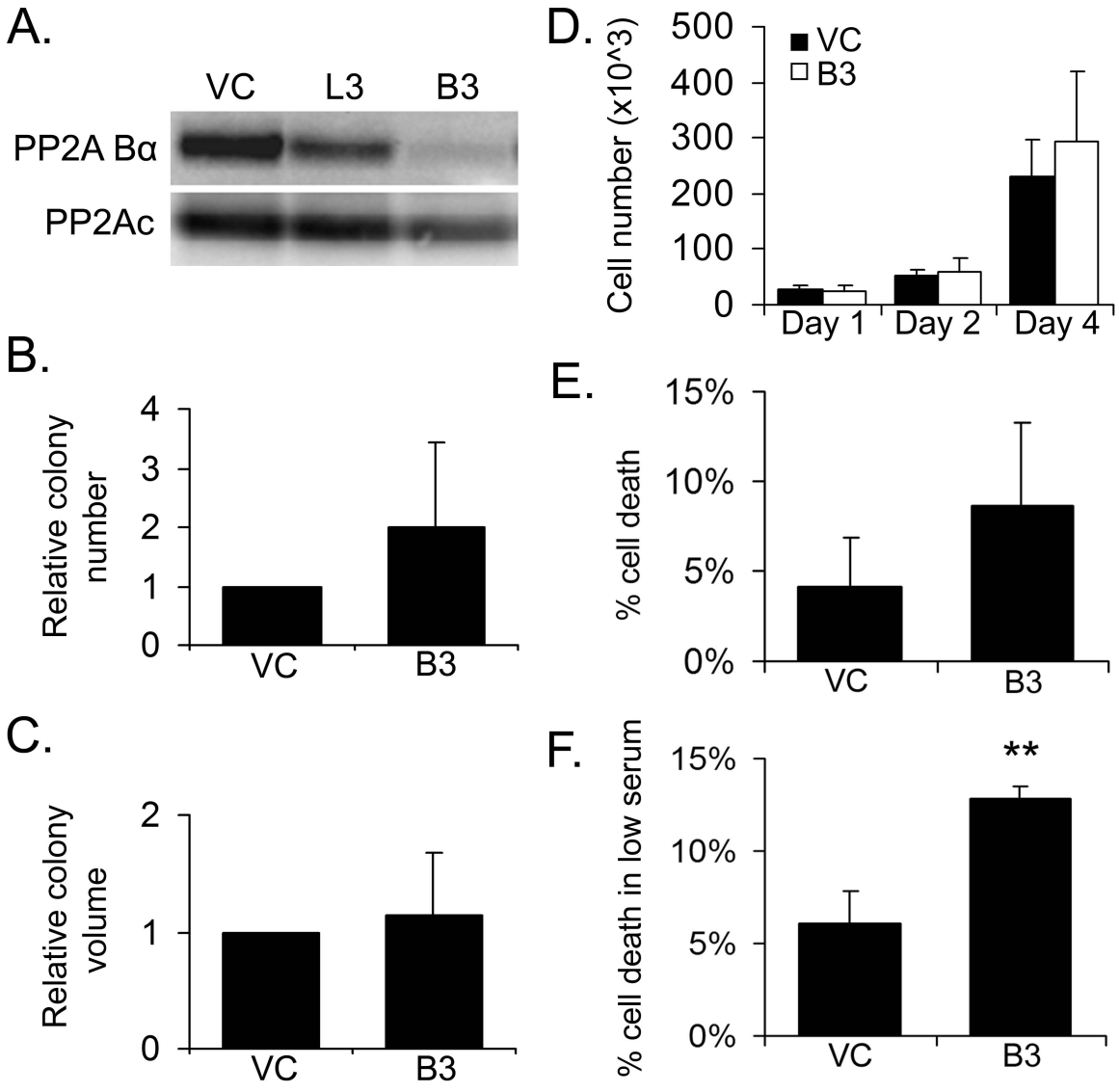


Figure 18. Knocking down Bα does not significantly enhance transformation. (A) Knockdown of Bα in HEKTERASB56γ cells. HEKTERASB56γ cells stably expressing empty pLKO.1 vector control (VC) or Bα shRNA (B3) were lysed and Bα levels were detected by western blotting. PP2A C subunit (PP2Ac) was used as loading control. Additionally, L3 LCMT-1 knockdown cells were also included to show that Bα expression is reduced when PP2Ac methylation is downregulated indicating that Bα is methylation-sensitive. (B and C) Anchorage-independent growth of VC and B3

knockdown cells in soft agar. Average colony numbers (B) and average colony volumes (C) were determined and data is shown in graphs as fold change relative to VC. Error bars in B-C represent S.D. of three independent experiments performed in triplicate. No statistically significant changes were observed. (D) Growth rates of adherent VC and B3 knockdown lines were assessed over a 4-day period and graph represents the average number of live cells at each time point. (E) The VC and B3 knockdown lines were assessed for death rate after 3 days in normal culture conditions and the average percentage of dead cells of the VC and B3 knockdown lines is presented in the graph as % cell death. (F) VC and B3 were grown in low serum for 48 hours and assessed for death rate. The average percentage of dead cells in each line is presented in the graph. Error bars in D-F represent S.D. of three experiments. **, $P \leq 0.01$.

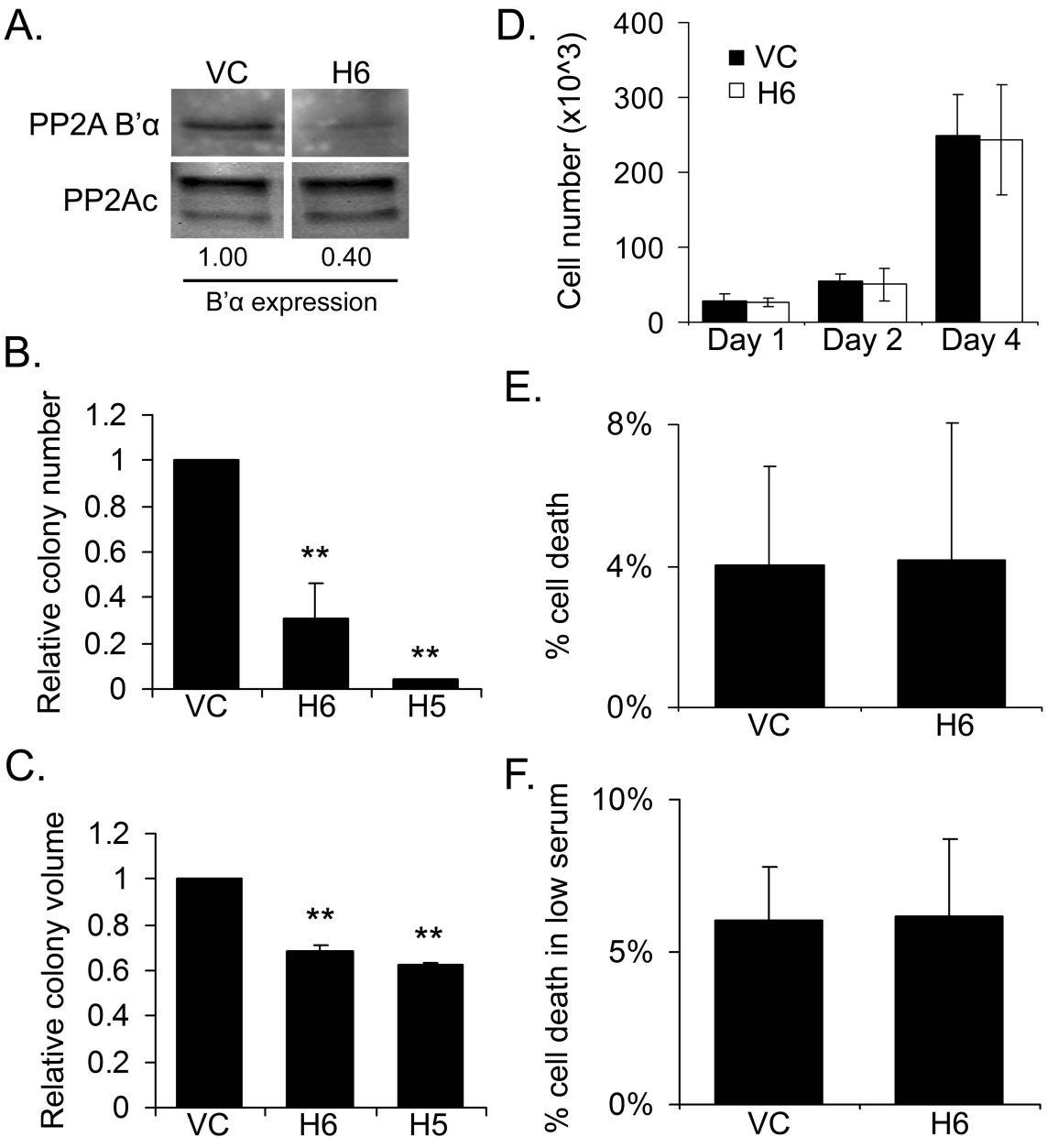


Figure 19. Knocking down B'α reduces transformation. (A) Knockdown of B'α in HEKTERASB56γ cells. HEKTERASB56γ cells stably expressing empty pLKO.1 vector control (VC) or B'α shRNA (H6) were lysed and B'α levels were detected by western blotting. PP2A C subunit (PP2Ac) was used as loading control. (B and C) Anchorage-independent growth of VC and B'α knockdown (H6 and H5) cells in soft agar. Average

colony numbers (B) and average colony volumes (C) were determined and data is shown in graphs as fold change relative to VC. Error bars in B-C represent S.D. of three independent experiments performed in triplicate. ** $P \leq 0.01$. (D) Growth rates of adherent VC and H6 knockdown lines were assessed over a 4-day period and graph represents the average number of live cells at each time point. (E) The VC and H6 knockdown lines were assessed for percentage death after 3 days in normal culture conditions and the average percentage of dead cells of the VC and H6 knockdown lines is presented in the graph as % cell death. (F) VC and H6 were grown in low serum for 48 hours and assessed for the percentage of dead cells. The average percentage of dead cells in each line is presented in the graph. Error bars in D-F represent S.D. of three experiments. No statistically significant changes were observed.

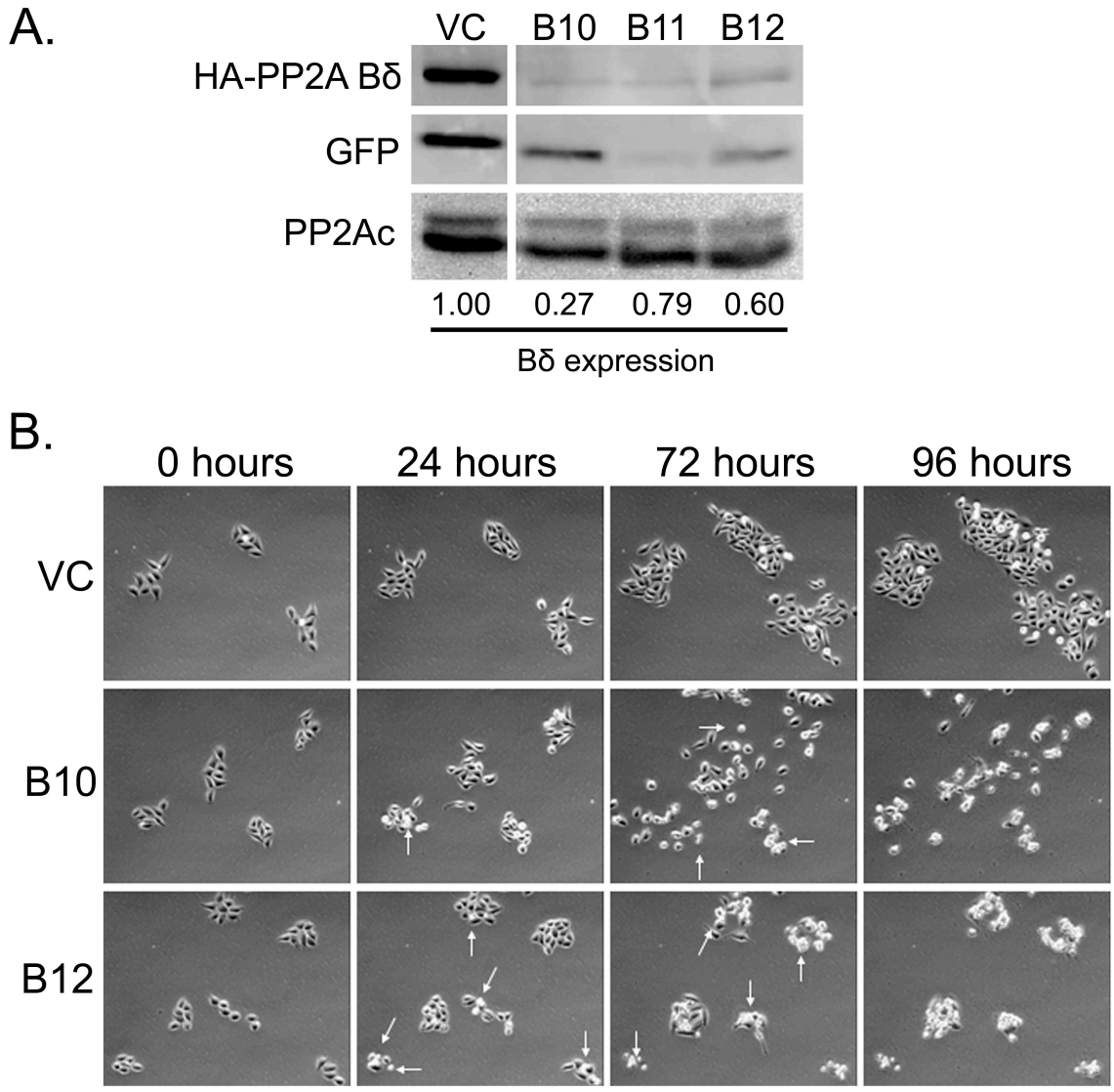
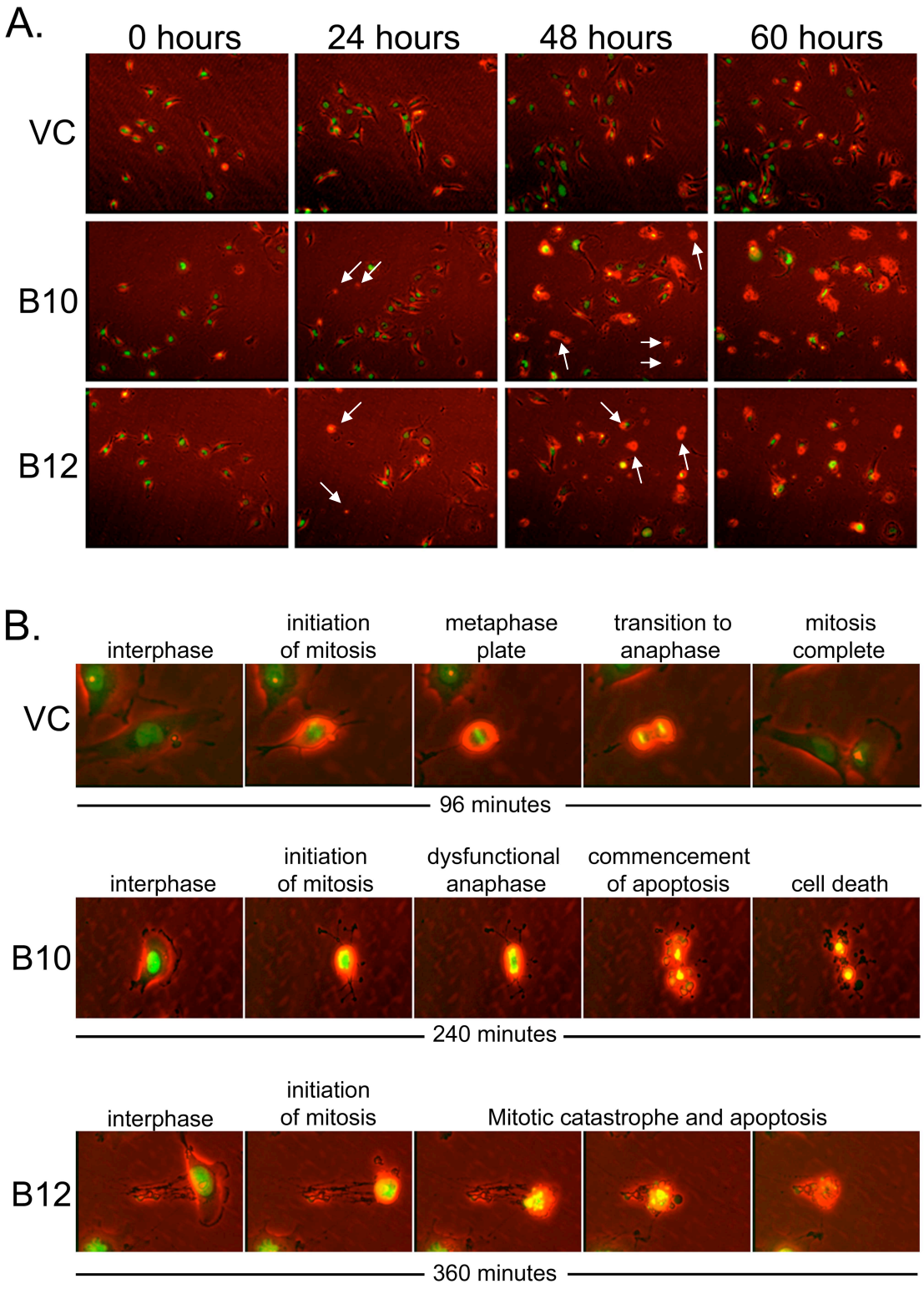


Figure 20. Knocking down Bδ in HeLa cells causes widespread death. (A) Knockdown of Bδ in HeLa cells. HeLa cells expressing empty pLKO.1 vector control (VC) or Bδ shRNAs (B10-B12) were cotransfected with HA-tagged PP2A-Bδ and GFP to report transfection efficiency. Forty-eight hours post-transfection, cells were lysed and HA-Bδ and GFP levels were detected by western blotting. PP2A C subunit (PP2Ac) was used as loading control. Reductions in Bδ expression were quantitated by normalizing the Bδ signaling to GFP and then normalizing to the loading control, PP2Ac. (B) HeLa cells

were plated on 3.5 cm plates and infected overnight (MOI of 3) with VC, B10 or B12 lentivirus. Forty-eight hours post-infection, infected HeLa cells were observed by phase-contrast, time-lapse microscopy for 4 days. Representative frames at 0 hours, 24 hours, 72 hours, and 96 hours are depicted to illustrate the differences in growth and survival between the VC cells and the B10 and B12 knockdown cells.


A.
0 hours
24 hours
48 hours
60 hours
VC
B10
B12
B.
interphase
initiation of mitosis
metaphase plate
transition to anaphase
mitosis complete
VC
96 minutes
interphase
initiation of mitosis
dysfunctional anaphase
commencement of apoptosis
cell death
B10
240 minutes
interphase
initiation of mitosis
Mitotic catastrophe and apoptosis
B12
360 minutes

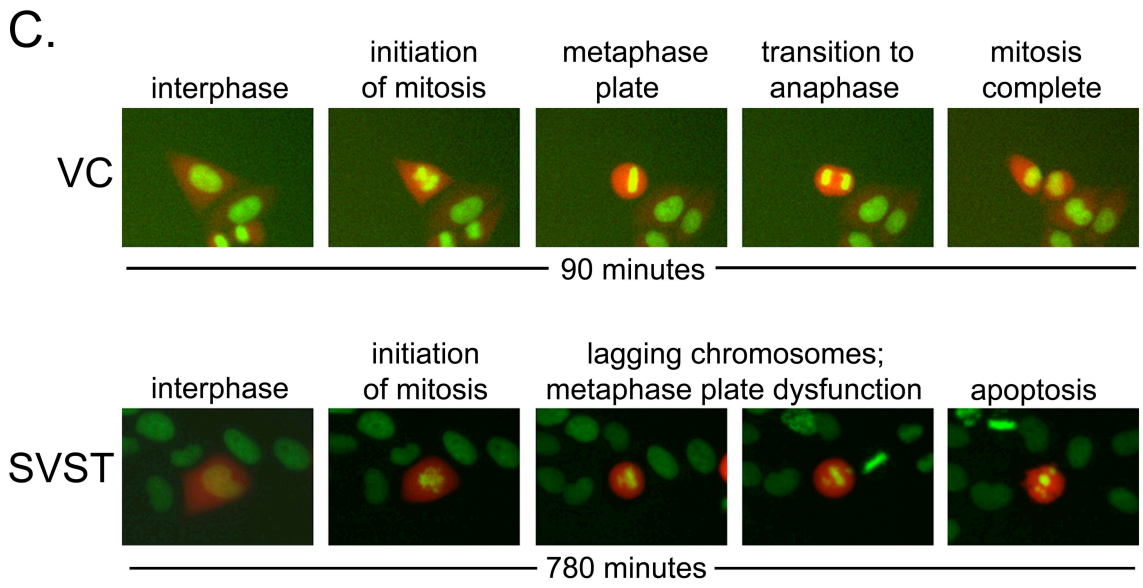


Figure 21. Bδ knockdown or SV40 small T (SVST) expression in HeLa cells causes mitotic defects and apoptosis. (A) H2B-GFP cells infected with VC, B10 and B12 lentivirus were observed by fluorescence (UV), time-lapse microscopy, 24 hours post infection for 60 hours. Individual frames at 0 hours, 24 hours, 48 hours and 60 hours represent changes seen during the 2.5 day time course. Red represents phase and green represents GFP-H2B fluorescence on DNA. (B) A zoomed in view of a representative cell undergoing mitosis in the VC, B10, and B12 cells in Figure 21A is provided to highlight the dysfunctional mitotic events occurring in Bδ knockdown cells. (C) H2B-GFP cells transfected with control vector mCherry or cotransfected with SV40 small T (SVST) and mCherry were observed by fluorescence (UV), time-lapse microscopy, 48 hours post-transfection. A representative cell undergoing mitosis in control and SVST-expressing cells is shown to highlight differences in mitotic events and apoptosis. Red represents mCherry positive transfection control and green represents GFP-H2B fluorescence on DNA.

CHAPTER 3.3

Results

Sustained PP2Ac Methylation Enhances the Tumor Suppressive Function of PP2A through Down-regulation of the Akt Pathway

Hypothesis: Down-regulation of PP2Ac methylation promotes transformation; therefore, downregulating the PP2A methylesterase, PME-1 will support methylation and reduce cellular transformation.

Predictions: 1) shRNA-directed knockdown of PME-1 will reduce cellular transformation and 2) will do so by reducing Akt and S6 kinase activation, pathways affected by modulating LCMT-1.

Suppression of PME-1 Reduces Transformation

Suppression of PP2Ac methylation by reducing LCMT-1 results in enhanced transformation in HEKTERASB56γ cells; therefore, the first prediction of my hypothesis is that enhancing PP2Ac methylation by decreasing the level of PME-1, the methylesterase responsible for PP2Ac demethylation, would reduce transformation. To test this prediction, HEKTERASB56γ cells were infected with vector control (VC) or PME-1-directed (P2 or H5) shRNA viruses, and Western blot analysis was used to verify the knockdown of PME-1 (Figure 22A). Quantitation of four independent experiments showed that both the P2 and H5 PME-1 shRNAs stably reduced PME-1 expression by 76% ± 15% ($P<0.001$) and 77% ± 24% ($P<0.001$), respectively, compared with the VC

cells. To ensure that at least one of the PME-1-directed shRNAs was affecting PP2Ac methylation, the steady-state level of PP2Ac methylation in VC and P2 lysates was measured by Western analysis (Figure 22B). As can be seen by comparing the minus base (-) lanes (which show endogenous levels of unmethylated PP2Ac) in Figure 22B, the amount of unmethylated PP2Ac was reduced in P2 PME-1 knockdown cells compared with the VC control cells. Quantitation of three experiments showed that 70% of total PP2Ac was methylated in VC cells whereas PME-1 (P2) knockdown cells had 83% of total PP2Ac methylated, a significant increase from VC cells (Figure 22C). Thus, PME-1 knockdown increased steady-state PP2Ac methylation. To examine the role of PME-1 in transformation, VC and P2 cells were evaluated for anchorage-independent growth in soft agar. After 3 weeks of growth in agar, colonies were examined by microscopy (Figure 22D) and quantitative analysis showed that P2 cells had a significant 78% and 56% reduction in colony number and volume, respectively (Figures 22E and F). Additionally, the second PME-1-targeting shRNA (H5) was used to confirm the reduction in transformation. Soft agar analysis revealed that PME-1 knockdown (H5) also significantly reduced colony number (by 67%) and colony volume (by 27%) (Figure 22D-F). These results strongly support the hypothesis that promoting PP2Ac methylation inhibits cellular transformation and is consistent with the idea that methylation promotes the tumor suppressive function of PP2A.

PME-1 Knockdown Does Not Affect Proliferation Rate or the Akt and p70/p85 S6K Signaling Pathways during Normal Anchorage-Dependent Growth

To dissect how a reduction in PME-1 reduces anchorage-independent growth, P2 cells were analyzed for differences in proliferation and in the Akt and S6K signaling pathways during anchorage-dependent growth. Comparison of the proliferation rates of VC and P2 cells over a 4-day period showed no significant differences between the two cell lines, indicating that PME-1 knockdown does not alter cell proliferation in anchorage-dependent conditions (Figure 23A). No obvious differences were seen in the number of dead cells while counting cells for the proliferation assay, therefore, it was assumed that an increase in death would not be detected and, therefore, would not contribute to the reduced transformation seen in anchorage-independent conditions. To assess whether PME-1 knockdown inactivates progrowth/prosurvival signaling pathways during anchorage-dependent growth, lysates from VC and P2 cells were probed for the levels of Akt activating phosphorylations, T308 and S473, as well as the downstream Akt substrate, GSK3β. Activating phosphorylation levels of rpS6 were also examined as a readout of S6K pathway activation. No differences in the phosphorylation levels of these proteins were seen indicating that PME-1 knockdown did not alter Akt or rpS6 activation (Figure 23B). These results show that the activation state of these signaling molecules is not altered by PME-1 knockdown in adherent cultures, consistent with the lack of effect of PME-1 knockdown on proliferation during normal anchorage-dependent growth.

PME-1 Knockdown Downregulates the Akt and p70/p85 S6K Pathways in Anchorage-Independent Conditions

Taking into the consideration that the biochemical effects of LCMT-1 knockdown were only revealed upon analyzing lysates from anchorage-independent suspension cells, it became clear that the same mechanism was likely occurring in these cells. Therefore, VC and PME-1 knockdown cells (P2) were plated equally on ultra-low-adherence tissue culture plates and grown for 1 week in anchorage-independent conditions. After 1 week, suspension cells were collected and weighed to observe changes in anchorage-independent growth and P2 cells showed a significant 38% decrease in total weight, thus indicating that these cells are at a selective disadvantage compared to VC cells (Figure 24A). This finding confirmed the hypothesis that the negative effects of PME-1 knockdown on growth and survival are specific for anchorage-independent conditions. Therefore, lysates from the 1 week old suspension VC and P2 cells were analyzed by Western blot for changes in the Akt pathway, a pathway activated by LCMT-1 knockdown as established in Chapter 3.1. Relative to VC cells, P2 cells had significantly reduced activated Akt as indicated by decreased phosphorylation of both T308 and S473 by 45% (Figure 24B and C). Although the Akt substrate, GSK3β, was not significantly reduced (Figure 24B and C) in the suspension lysates, clear reductions in other Akt substrates were revealed by probing with a phospho-Akt substrate antibody (Figure 24D). Of note, PME-1 knockdown reduced the phosphorylation of several proteins migrating at positions of proteins whose phosphorylation was increased in LCMT-1 knockdown cells, including the bracketed rpS6 (compare Figure 25D to Figure 9D, arrows and bracket). This result supports the idea that loss of PME-1 regulates the phosphorylation of some of

the same proteins via Akt that were altered during LCMT-1 knockdown. Lastly, immunoblot analysis with phospho-rpS6 and total rpS6 antibodies confirmed that PME-1 knockdown reduces rpS6 phosphorylation by 72% ± 16% (p-value = 0.002) (Figure 24E) indicating that the S6K pathway is likely being downregulated. Overall, these results support the idea that PP2Ac methylation drives PP2A's tumor suppressive function and suggests that supporting methylation through targeting PME-1 may be relevant as an anti-cancer therapeutic strategy.

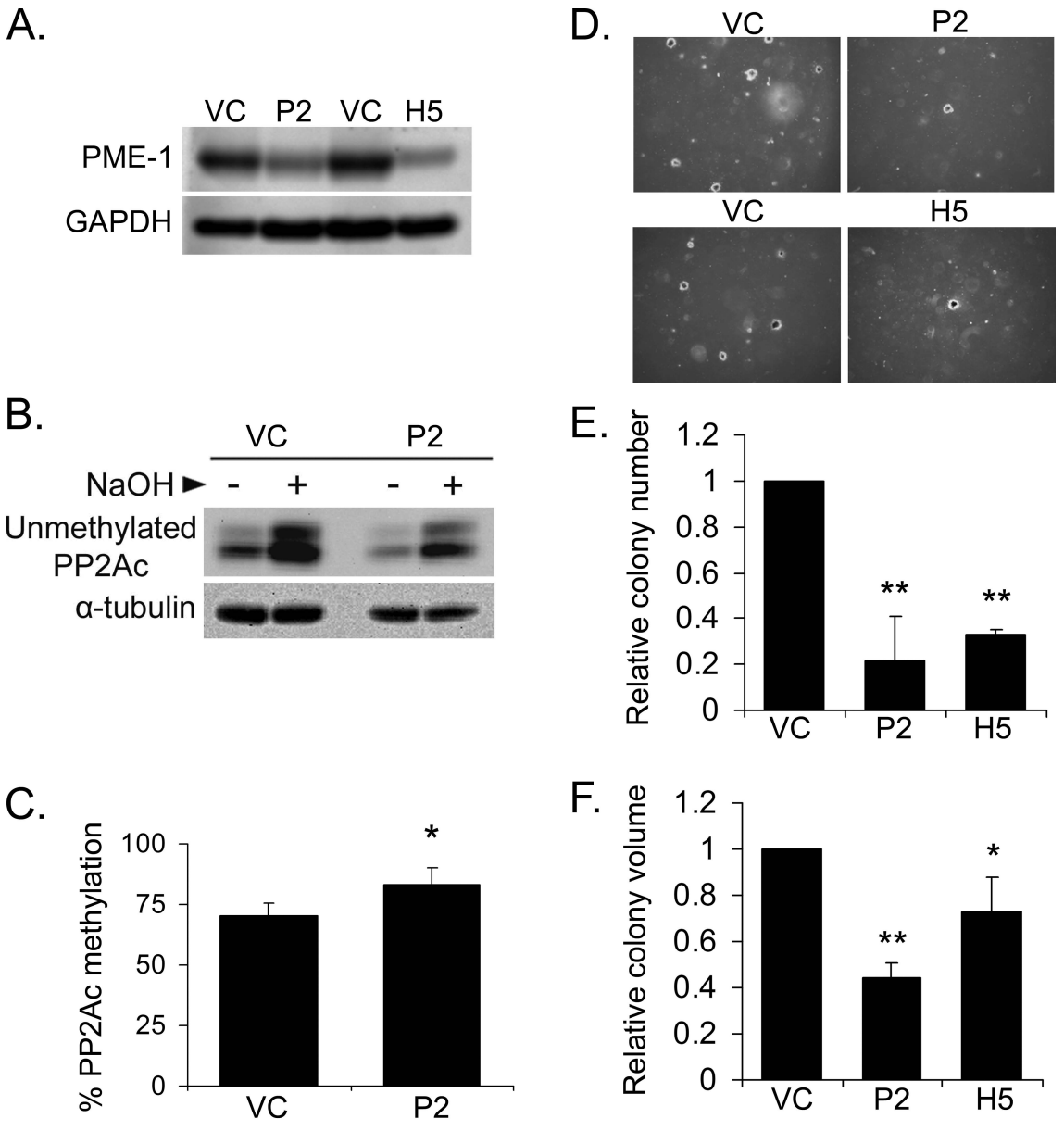


Figure 22. Knocking down PME-1 reduces transformation. (A) Knockdown of PME-1 in HEKTERASB56γ cells. HEKTERASB56γ cells stably expressing empty pLKO.1 vector control (VC) or PME1 shRNAs (P2 and H5) were lysed and PME-1 levels were detected by western blotting. GAPDH was used as loading control. (B and C) PP2Ac becomes more methylated in the PME-1 knockdown line. (B) Equal volumes of lysates

from VC and P2 cells were either treated with preneutralized base solution (- lanes; show unmethylated PP2Ac levels in cells) or base treated and then neutralized (+ lanes; 100% demethylated controls) prior to being analyzed by western blotting for the level of unmethylated PP2Ac and α-tubulin (loading control). (C) Graph depicts the average percent methylation of PP2Ac in the lysates, calculated as described in the legend to Fig. 6C. Error bars represent S.D. of three independent experiments. *, $P \leq 0.05$. (D-F) Anchorage-independent growth of VC, P2, and H5 PME-1 knockdown cells in soft agar. (D) Photographs show small, single, representative fields within the agar wells. Average colony numbers (E) and average colony volumes (F) were determined and data is shown in graphs as fold change relative to VC. Error bars represent S.D. of three independent experiments performed in triplicate. *, $P \leq 0.05$; ** $P \leq 0.01$.

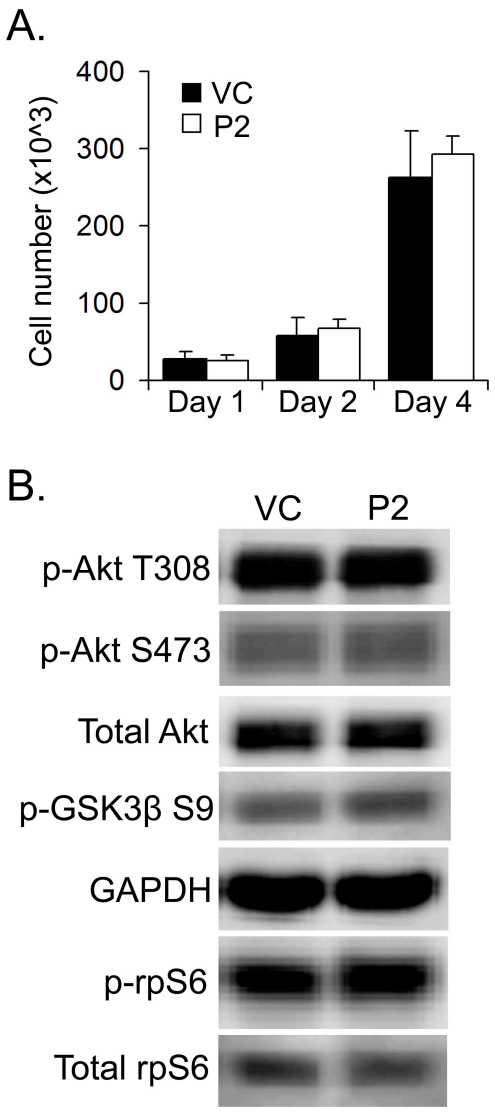


Figure 23. Adherent control and PME-1 knockdown lines show no difference in growth or Akt and S6K signaling. (A) Growth rates of adherent VC and P2 knockdown lines were assessed over a 4-day period and graph represents the average number of live cells at each time point. (B) Lysates from adherent VC and P2 knockdown cells were analyzed by western blotting for pAkt T308, pAkt S473, pGSK3β S9, and p-rpS6. GAPDH, total Akt, and total rpS6 were used as loading controls. No statistically significant changes were observed.

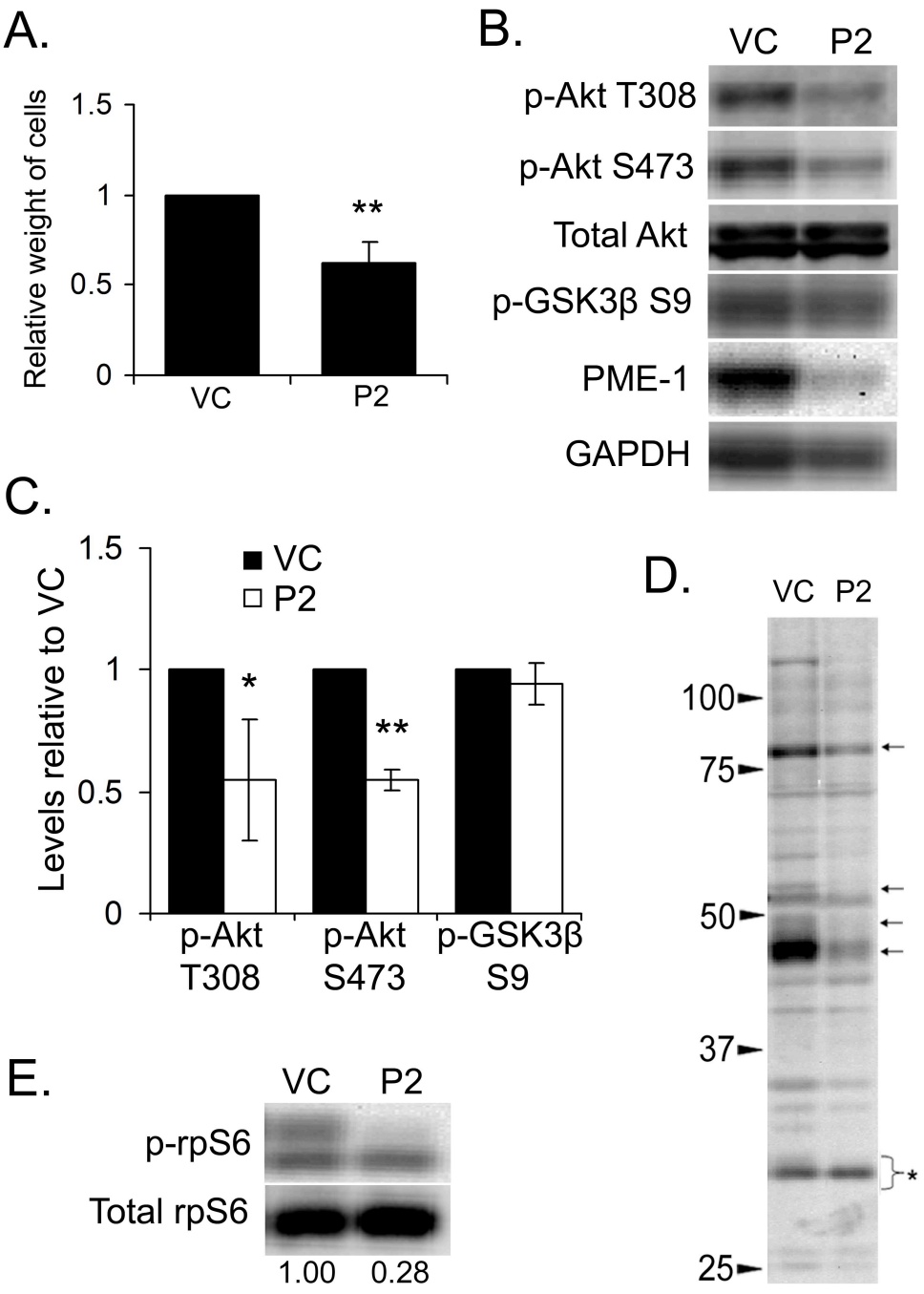


Figure 24. PME-1 knockdown downregulates the Akt and S6K pathways in anchorage-independent conditions. (A) Equal numbers of vector control (VC) and PME-1 knockdown (P2) cells were seeded on low-binding tissue culture dishes to analyze differences during anchorage-independent growth. After 1 week, suspension cells were

weighed to assess growth. In the graph, data is presented as average fold change relative to VC for three independent experiments. (B) Lysates from VC and P2 knockdown suspension cells were analyzed by western blotting for changes in activation of Akt. GAPDH and total Akt were used as loading controls. Western blotting for PME-1 confirmed the knockdown of PME-1 in suspension cultures. (C) Graph depicts the average levels of phospho-Akt T308, phospho-Akt S473 and phospho-GSK3β S9 in three independent immunoblot experiments as fold change relative to VC. Experiments were performed in triplicate and error bars in all panels represent S.D. *, $P \leq 0.05$; **, $P \leq 0.01$. (D) An anti-Akt phospho-substrate motif (R/K)X(R/K)XX(pT/pS) antibody was used to probe the VC and P2 knockdown suspension cell lysates. Arrows highlight some proteins with increased phosphorylation in the P2 knockdown cells relative to VC. Bracket with asterisk indicates phospho-rpS6, which is known to cross-react with this antibody. (E) Lysates were probed for changes in rpS6 phosphorylation. Total rpS6 was used as a loading control. Quantitation represents three experiments, p-value = 0.002.

CHAPTER 4

Discussion

Circumventing normal cellular control of PP2A by PP2Ac methylation is a general strategy for ST- and MT-mediated transformation. [220]

In Chapter 3.1, the results demonstrate that SVST and PyST, like PyMT, form heterotrimers with PP2A in a methylation-insensitive manner. In addition, reducing PP2Ac methylation by knocking down LCMT-1 or overexpressing PME-1 enhances progrowth and prosurvival signaling and promotes transformation in the genetically defined HEKTERASB56γ human cell system. Overall, these results support the hypothesis that methylation-insensitive viral B-type subunits, MT and ST oncoproteins, mediate transformation by replacing methylation-sensitive cellular PP2A B-type subunits to circumvent the antigrowth, antisurvival effects of methylation-sensitive PP2A heterotrimers. The fact that both polyomavirus and SV40 oncoproteins form PP2A heterotrimers independent of the methylation status of PP2Ac suggests that circumventing control by PP2Ac methylation is a general strategy for MT and ST-mediated transformation. Together, these results provide new insight into the mechanism of transformation by MT and ST oncoproteins and are consistent with the idea that a reduction in LCMT-1 amount or activity could contribute to human cancer.

LCMT-1 knockdown in HEKTERASB56γ cells increased both the number and size of the colonies observed in soft agar assays, indicating that loss of LCMT-1 causes additional signaling changes to those already present in the HEKTERASB56γ cells. Activation of Akt, which was demonstrated by both direct measurement of activating

phosphorylations and analysis of downstream signaling, was required for LCMT-1 knockdown-induced transformation. Phosphorylation of Akt T308 was particularly increased on LCMT-1 knockdown, suggesting that this phosphorylation site may be regulated directly or indirectly by one or more methylation-sensitive PP2A B-type subunits. Consistent with this possibility, the highly methylation-sensitive PP2A Bα subunit has been reported to direct the dephosphorylation of Akt T308 [61]. Another possible candidate is B′β, which has been reported to negatively regulate Akt T308 phosphorylation and seems to be methylation-sensitive [52, 73]. Akt S473 phosphorylation, however, is regulated by PP2A B′γ (B56γ) [83], which is knocked down already in the HEKTERASB56γ cells. LCMT-1 knockdown further increases phosphorylation at this site, suggesting that LCMT-1 may regulate additional PP2A heterotrimeric forms that target Akt S473. Because B′β has also been reported to promote dephosphorylation of Akt S473 [73], it is a possible target for LCMT-1-mediated effects. Bα and B′α, which are both highly methylation-sensitive [44, 52], do not seem to be candidates for modulation of Akt S473 phosphorylation because Bα or B′α knockdown does not affect phosphorylation on this site [61, 83].

LCMT-1 knockdown also activated p70/p85 S6K and increased phosphorylation of their downstream target, rpS6, on a site that correlates with increased translation [223, 224]. Given that S6K can be activated by Akt through the mTOR pathway [85, 225], much of the LCMT-1-dependent activation of the S6K pathway might be a consequence of Akt activation. Consistent with this possibility, dominant-negative Akt expression greatly reduced p70/p85 S6K activation observed on LCMT-1 knockdown. However, the small amount of residual phosphorylation on p85 S6K (P = .04 when compared with

control) and more substantial residual phosphorylation of rpS6 (P = .02 when compared with control) suggest there is also more direct, Akt-independent regulation of this pathway by methylation-sensitive PP2A heterotrimers. Consistent with this idea, Bα-containing PP2A heterotrimers have been reported to directly bind to and negatively regulate p70 S6K [94]. Furthermore, E4orf4, an adenovirus protein that inhibits the Bα-containing PP2A heterotrimers, increases the level of activated p70 S6K and inhibitory phosphorylation of 4EBP1 [226], thus likely promoting translation. Thus, whereas Akt-dependent signaling is required for LCMT-1 knockdown to enhance transformation in HEKTERASB56γ cells, methylation-sensitive PP2A heterotrimers regulate translation in both Akt-dependent and independent manners.

Interestingly, the importance of LCMT-1 for the regulation of Akt and p70/p85 S6K and increased growth and/or survival was revealed in the current study only under anchorage-independent growth conditions. This finding is significant considering that anchorage-independent growth facilitated by resisting anoikis, programmed cell death induced by detachment of cells from their extracellular matrix (ECM), is a critical step in cancer initiation and progression [222]. Under anchorage-dependent conditions, growth and survival signaling are normally initiated by the interaction of integrins with the ECM. When cells detach from the substratum, these signals are abrogated, and cells undergo growth arrest and anoikis. Cancer cells resist anoikis by aberrant ECM-independent growth and survival signaling and can proliferate and survive in anchorage-independent conditions. Inhibition of methylation-sensitive PP2A heterotrimers by LCMT-1 knockdown may provide prosurvival and progrowth signaling normally initiated by the interaction of integrins with the ECM. Consistent with this idea, PP2A is involved in

integrin-mediated growth and survival signaling [184, 187, 227-232]. Also, Src, a kinase that phosphorylates and inactivates PP2A [233], functions in prosurvival integrin signaling [222]. Importantly, inhibition of PP2A is known to cause increased activation of Akt and p70 S6K [226, 231, 234, 235]. Therefore, I propose that LCMT-1 knockdown promotes anchorage-independent growth by reducing methylation-sensitive PP2A complexes that normally function to promote growth arrest and anoikis through inactivation of Akt under anchorage-independent conditions. Because SVST targets methylation-sensitive B-type subunits for replacement and evades control of heterotrimer formation by PP2Ac methylation, the ability of SVST expression to induce HEKTER cells to grow in an anchorage-independent environment may be explained in part by these findings.

In contrast to previous results with HeLa cells where LCMT-1 knockdown induced apoptosis in a small portion of the cells [52, 53], no increase in cell death on LCMT-1 knockdown was seen in the current study. The cell death in HeLa cells was reported to be due at least in part to mitotic checkpoint errors [31]. Therefore, it would not be unreasonable to expect that, with strong prosurvival signaling, these errors might not lead to death. Thus, differences in prosurvival signaling between HeLa and HEKTERASB56γ may explain this disparity. Consistent with this possibility, the Pallas lab has found that LCMT-1 knockdown increases apoptosis in some cancer cell lines (e.g., HeLa and H1299 cells) but not others ([53] and unpublished data).

PME-1 overexpression also reduced PP2Ac methylation, enhanced transformation, and caused many of the same signaling effects as LCMT-1 knockdown, further supporting the hypothesis that reduction in PP2Ac methylation enhances

transformation through up-regulation of Akt and p70/p85 S6K pathways. The reduced potency of overexpressing PME-1 compared to LCMT-1 knockdown may result from PME-1 overexpression having other effects on PP2A holoenzyme assembly or activity. This possibility is consistent with the report that PME-1 functions in PP2A biogenesis [24, 236], and with the finding that PME-1 knockout in mice results in reduced PP2A activity toward a phosphopeptide substrate and reduced total PP2A protein in several tissues [237]. Consistent with my findings that PME-1 over-expression promotes transformation, PME-1 was found to be over-expressed in human gliomas and knockdown of PME-1 in HeLa cells reduces anchorage-independent growth in soft agar [238]. Together, these data argue strongly that loss of PP2A methylation promotes transformation.

Consistent with the idea that MT and ST oncoproteins transform cells in part by circumventing the normal control of PP2A by methylation, the Akt and p70/p85 S6K pathways activated by LCMT-1 knockdown are known to be activated by SV40- and polyomavirus-induced transformation [62, 185, 205, 208, 239-243]. PyMT, PyST, and SVST have all been reported to induce Akt phosphorylation on activating residues [62, 185, 205, 208, 239, 240], whereas PyMT and PyST have been shown to activate the p70 S6K pathway [62, 243]. Similar to my present findings, rpS6 phosphorylation induced by these viruses is likely mediated through both Akt-dependent and Akt-independent pathways. For example, PyMT was reported to cause p70 S6K activation and rpS6 phosphorylation through activation of PI3-kinase (and thus Akt) but also to directly induce rpS6 phosphorylation independent of PI3-kinase and p70 S6K [243]. More recent data indicate that PyST can also enhance rpS6 phosphorylation [62]. Therefore, it is

likely that MT and ST oncoproteins must target a specific subset of PP2A complexes that regulate these pathways in order to circumvent PP2A's tumor suppressive function.

Altogether, the results thus far suggest that the MT and ST antigens likely override several methylation-sensitive B-type subunits to block their antigrowth/antisurvival functions. In support of this idea, knockdown of B'γ in HEKTER cells only partially recapitulates the level of transformation observed in SVST-expressing cells [171] and knockdown of LCMT-1 boosts transformation further. This effect is likely due to the fact that LCMT-1 knockdown targets many, if not all, of the same B-type subunits as SVST, [98, 171, 173, 175] thereby promoting robust transformation. Expression of SVST promotes the activation of cell cycle machinery and antiapoptotic proteins and multiple progrowth/prosurvival pathways, including ERK/MAPK and Akt (reviewed in [172]), processes heavily regulated by PP2A complexes containing methylation-sensitive B-type subunits. Therefore, it is likely that SVST, as well as other T antigens, downregulate antigrowth/antisurvival signaling by circumventing cellular control of PP2A by methylation. For these reasons, one could speculate that suppression of additional methylation-sensitive B-type subunits in conjunction with B'γ will further enhance transformation. Lastly, the helper function of the PP2A-independent, N-terminal J domain of T antigens has not yet been fully understood with regards to its contribution to transformation; therefore, it is possible that this region may also support transformation when expressed in the HEKTERASB56γ system.

Expression of the PP2A-independent region of SVST does not complement B'γ knockdown in transformation

In Chapter 3.2, I hypothesized that expression of the PP2A-independent N-terminal region of SVST would provide helper function in conjunction with B'γ knockdown to further promote transformation in HEKTERASB56γ cells. Therefore, HEKTERASB56γ cells were infected with ST110-expressing lentivirus to express the PP2A-independent portion of SVST. Results from soft agar analysis demonstrated that expression of SVST's PP2A-independent region did not complement B'γ knockdown in this cell system but rather significantly reduced transformation. This finding was quite surprising considering the fact that most studies suggest that the N-terminal region of SVST provides additional functions that would have presumably enhanced transformation. First, the N-terminal portion of SVST, which shares a common amino-terminal exon with other T antigens, encodes for 82 amino acids, of which 70 amino acids represents a highly conserved J domain [244-247]. One well-established interaction of the T antigen J domain is its association with Hsp70, a family of heat shock proteins that modulate protein folding. Specifically, the J domain binds to and stimulates the ATPase activity of specific Hsp70 family members to promote cell survival by preventing protein aggregation and misfolding, controlling protein turnover and degradation, and promoting proper protein folding and oligomeric assembly [245, 248-250]. Thus, the J domain would be expected to promote the activation of progrowth, prosurvival molecules that rely on Hsp70 chaperone activity. Furthermore, Hsp70 is found to be highly expressed in various types of malignant tumors and also correlates with increased cell proliferation and poor therapeutic outcomes in breast cancer [251-

253]. Second, the PP2A-independent portion of SVST has been linked to the transactivation of cyclin A which acts to initiate DNA replication at S phase of the cell cycle [180]. Thus, expression of the PP2A-independent region promotes cell cycle progression and cell proliferation. Third, Affymetrix GeneChip analysis of whole genomes comparing HEKTER cells expressing full-length SVST and the N-terminal ST110, PP2A-independent region revealed that the N-terminal portion of SVST could regulate over 100 genes in a PP2A-independent manner [184]. Many genes affected by expression of ST110 were related to promoting cell proliferation and survival, thereby suggesting that the N-terminal region of SVST would provide additional signals in conjunction with B'γ knockdown to promote transformation.

The unexpected finding that ST110 reduced transformation in HEKTERASB56γ cells reveals that the PP2A-independent region does not complement suppression of PP2A complexes but rather, suppresses transformation when expressed in trans with knockdown of B'γ. In support of this finding, one study revealed that expression ST110 in HEKTER cells (without the B'γ knockdown) also reduced transformation albeit to a lesser, statistically insignificant extent [178]. These results suggest the possibility that ST110 may cause effects that may not be biologically relevant but, regardless, offset the effects of knocking down PP2A complexes. Alternatively, expression of the PP2A-independent region of SVST may elicit a dominant negative effect and could possibly lead to its dysfunction when not expressed in cis with its C-terminal, PP2A-targeting region. One could speculate that the presence of the J domain of ST110 alone may act in a dominant negative manner to disrupt the normal interactions with the Hsp70 chaperone proteins and in fact may act as an inhibitor of Hsp70 activity. In support of this

supposition, recent research has shown that upregulation of Hsp70 is found in various human tumors and correlates with increased chemotherapy resistance and poor prognosis [254, 255]. Furthermore, newly developed inhibitors of Hsp70 and other heat shock proteins have shown promise as anticancer therapies [256, 257]. Thus, ST110 may indeed be acting as an inhibitor of Hsp70 function; therefore, it would be interesting to determine the effects of expressing this PP2A-independent region of SVST on chaperone function, protein folding and overall cell growth and survival.

Suppression of methylation-sensitive, SVST-targeted PP2A B-type subunits in combination with B'γ knockdown reveals differential effects on cell growth, survival, and transformation

Expression of DNA tumor virus MT and ST antigens has been implicated in promoting transformation though the deregulation of PP2A-modulated processes including the cell cycle, cell proliferation, survival, and apoptosis (reviewed in [172]). SVST, like PyST and PyMT, blocks PP2A function by replacing certain B-type subunits resulting in transformation. Because only a subset of the PP2A-mediated cellular processes and pathways are altered during MT and ST expression and because complete inhibition of PP2A is lethal, these T antigens must target only a specific population of PP2A holoenzymes [14, 187]. Specifically, Bα, B'α, and B'γ, have been identified as being targeted for replacement by MT and/or ST oncoproteins [98, 171, 173, 175]. Interestingly, of the three cellular B-type subunits reported to be displaced by the MT or ST oncoproteins, two (Bα and B'α) are methylation-sensitive and one (B'γ) is likely to be methylation-sensitive [43, 44, 46, 52, 216]. Furthermore, based on the high sequence

homology between Bα and Bδ [190], it likely that Bδ is also methylation-sensitive and targeted by MT and ST oncoproteins. In Chapter 3.1, I investigated the effects of inhibiting PP2Ac methylation by targeting the PP2Ac methyltransferase, LCMT-1, and determined that suppression of PP2Ac methylation further enhanced cellular transformation in conjunction with B'γ knockdown. This suggests that MT and ST may promote transformation by circumventing methylation by targeting PP2A complexes containing methylation-sensitive B-type subunits. Therefore, in order to understand the individual and distinct contributions of each methylation-sensitive B-type subunit, the effects of Bα, Bδ, and B'α subunit knockdown were analyzed.

Knockdown of the PP2A Bα subunit in HEKTERASB56γ cells did not significantly increase either the number or the volume of colonies in soft agar and thus did not contribute to enhancing transformation as expected in this system. This result was particularly surprising due to the fact that PP2A-Bα complex formation relies considerably on the methylation of PP2Ac as can be seen by the reduction of Bα expression level in LCMT-1 knockdown cells. Worth mentioning, however, is the fact that a statistically insignificant 2-fold increase in colony number was observed on average in three independent assays upon Bα knockdown. The significance of this result was negatively affected due to a high level of variation among the three assays which yielded a large standard deviation. Interestingly, knocking down LCMT-1 produced a significant two-fold increase in colony number, a result quite consistent with knocking down Bα. Therefore, it is possible that reducing Bα drives this system towards a more transformed state but is not sufficient on its own to enhance transformation. In support of this notion, some studies have shown that suppressing Bα in HEKTER cells (without the

B'γ knockdown), does not promote transformation even though it is a known target of SVST [83, 171]. Thus, while SVST can target Bα and B'γ, other PP2A B-type subunits are likely being replaced, thus accounting for robust transformation when SVST is expressed.

To further understand why Bα did not significantly enhance transformation, adherent cell growth and death rates were assessed to determine if knocking down Bα affected proliferation and survival. No significant change in growth was observed and while a two-fold increase in death was seen, the result was not statistically significant. The insignificant increase in cell death did suggest, however, that the Bα knockdown cells may be generally weaker than the control cells. In support of this finding, another study found that knocking down Bα in HeLa cells caused increased cell death under normal conditions [53] indicating that Bα-containing complexes are involved in prosurvival responses. This may be related to the finding that Bα-containing PP2A complexes positively regulate MAPK pathway by activating the upstream kinase, Raf [97]. Although knocking down Bα in HEKTERASB56γ cells did not reveal a significant increase in death, challenging the cells by growing them in low nutrient conditions did demonstrate that these cells were at a selective disadvantage compared to control cells, with a statistically significant two-fold increase in death. This result suggests that Bα expression is important in regulating prosurvival pathways during nutrient deprived conditions. Therefore, one possible explanation for the lack of enhanced transformation is that nutrient levels in the soft agars may have not been adequate to fully support growth of the Bα knockdown cells. Although soft agars are fed once per week with serum-containing media during the three week assay, serum levels may have fallen to a level

that Bα knockdown cells cannot tolerate thereby mitigating their ability to grow in anchorage-independent conditions.

The fact that knocking down Bα does not enhance transformation is somewhat unexpected considering the established roles of Bα in the negative regulation of progrowth, prosurvival processes. Bα-containing PP2A complexes are known to dephosphorylate and reduce activation of Akt and p70 S6K1 thereby negatively regulating growth and survival signaling [61, 75, 94]. Considering that knocking down LCMT-1 enhanced Akt and p70 S6K1 phosphorylation, it was possible that this was due to reducing methylation-sensitive Bα-containing PP2A complexes. Although this may still be the case, there are possibly other methylation-sensitive B-type subunits that provide the phosphatase activity towards Akt and S6K1 to contribute to the enhanced transformation seen in LCMT-1 knockdown cells. Furthermore, PP2A-Bα also dephosphorylates ERK thereby downregulating the MAPK pathway, a pathway upregulated by SVST expression, and thus proliferation [69]. Therefore, reducing Bα would presumably cause MAPK upregulation promoting enhanced anchorage-independent growth.

There are, however, multiple functions of Bα that may explain the lack of enhanced transformation. Bα-containing PP2A complexes have been linked to the dephosphorylation of Raf on an inhibitory site to activate the MAPK pathway; therefore, knocking down Bα may reduce MAPK activation [97]. Additionally, Bα-containing PP2A holoenzymes are found to directly interact and dephosphorylate β-catenin to activate Wnt-mediated transcription [84]; hence, reducing Bα may abrogate Wnt signaling. Finally, PP2A-Bα is heavily involved in cell cycle regulation and depletion of

Bα has been linked to mitotic dysfunction [53, 111, 119, 226]. Therefore, one theory for why knocking down Bα did not enhance transformation is that knocking down Bα in this system may modulate multiple pathways which offset one another to produce no net effect on growth or survival. One could speculate that activating a progrowth/prosurvival pathway may be counterbalanced by the concomitant deactivation of another pathway, thereby having little effect on transformation. Furthermore, Bα shares high homology and many functions with Bδ; therefore, Bδ may be able to partially compensate for the reduction of Bα thereby impeding transformation. Overall, these results indicate that reducing Bα does not effectively contribute to transformation in conjunction with B'γ knockdown, but does not eliminate the possibility that it acts synergistically with other B-type subunits to produce the level of transformation observed in LCMT-1 knockdown or SVST expressing cells.

To further evaluate the hypothesis that knocking down methylation-sensitive B-type subunits in conjunction with B'γ will contribute to transformation and better recapitulate the level of transformation observed when LCMT-1 is reduced, cells knocked down for B'α were evaluated. Surprisingly, knockdown of the PP2A B'α subunit in HEKTERASB56γ cells using two different shRNAs significantly reduced both the colony number and volume. Due to the fact that PP2A-B'α complex formation relies considerably on the methylation of PP2Ac [216], this finding was unanticipated. Furthermore, one study found that reduction of B'α promoted transformation in HEKTER cells (without the B'γ knockdown) similar to the level of transformation observed when B'γ was knocked down [83]. Therefore, this evidence strongly suggested that reducing B'α would enhance transformation. One possibility for the disparity

between the two findings could be accounted for by the use of different shRNAs, as the two utilized in this study are distinct from those used in the study above that found that reducing B'α enhanced transformation. The finding here, however, did suggest the possibility that reducing B'α in moderately transformed cells could be effective to reduce anchorage-independent growth and survival. Therefore, B'α knockdown cells were assessed for adherent cell growth and death rates to determine if reduced transformation could be attributed to changes in proliferation or survival. Results from these assays revealed that cell proliferation and survival were not affected in adherent cultures, indicating that the effects of reducing B'α are specific for anchorage-independent growth.

The fact that knocking down B'α could not enhance transformation is somewhat unexpected considering the established roles of B'α in the negative regulation of growth, survival, and the cell cycle. First, B'α is a known phosphatase for the oncogenic c-myc protein; therefore, reducing B'α would be expected to increase c-myc stability [192]. B'α is also linked to inhibiting the progrowth, prosurvival β-catenin protein and the antiapoptotic protein, Bcl-2 [82, 134]. Furthermore, B'α is involved in protecting sister chromatid separation during the spindle checkpoint [116]. Therefore, suppressing B'α was expected to promote growth, survival and cell cycle progression, thus enhancing transformation. Although there is much evidence establishing B'α as a negative regulator of growth and survival, revealing the addition functions of the subunit is still in its early stages. In support of a positive role for B'α, one study found that knocking down B'α in HeLa cells resulted in increased apoptosis thereby implicating the subunit in a prosurvival response [258]. Therefore, it is possible that B'α positively mediates one or more progrowth/prosurvival signaling pathways and therefore reduces transformation

when B'α is knocked down. Alternatively, knocking down B'α may have allowed the remaining B'γ not knocked down in the system to assemble into the newly accessible PP2A A/C dimers to perform antigrowth/antisurvival functions, thereby reducing transformation. Regardless of the cause, these findings are quite interesting considering that suppression of B'α reduced growth and/or survival exclusively under anchorage-independent conditions. It is possible that suppressing B'α may modulate the cell in such a way that is not yet fully understood which could counteract oncogenic triggers in certain cell types. Therefore, it would be worthwhile to study the effects of knocking down B'α in various cancer lines to determine its therapeutic potential.

Finally, to evaluate the effects of knocking down Bδ, a subunit likely to be methylation-sensitive, Bδ-targeting lentivirus was produced for infection of the HEKTERASB56γ cell line. However, during the process of titering, HeLa cells infected with the Bδ-targeting lentivirus demonstrated a dramatic amount of cell death within one week post-infection. Therefore, to understand how reduction of Bδ promoted cell death, HeLa cells expressing fluorescently tagged histone H2B-GFP were infected with Bδ-targeting lentivirus to observe mitotic progression. Compared to cells infected with empty vector lentivirus, Bδ knockdown cells could not successfully progress through mitosis and eventually underwent apoptosis due to their inability to capture chromosomes, their abnormal chromosomal segregations, or their failure to properly exit mitosis. This finding is well supported by the literature and confirms that Bδ is critical for mitotic decisions. As stated in Chapter 1, Bδ is involved in mitotic entry by regulating the proteins involved in MPF activation, in chromosome segregation by preventing premature sister chromatid separation, and mitotic exit [104, 117, 120, 259]. Therefore, in Bδ knockdown cells,

enhanced death in cells that did commence mitosis was likely a result of uncontrolled, increased MPF activation, premature DNA segregation (as can be seen by lagging chromosomes), and/or faulty mitotic exiting, thereby triggering the apoptotic response. Interestingly, in the late, post-infection stage, many Bδ knockdown cells would not even initiate mitosis, would arrest, and eventually would die. This is likely due to the fact that their prior cell divisions had produced multiple irreversible and detrimental abnormalities which caused the cells to arrest and undergo apoptosis.

Of note, Bδ knockdown cells that were exposed to ultraviolet (UV) to visualize the GFP-tagged DNA (Figure 21A) appeared to commence apoptosis more rapidly than the non-UV exposed cells (Figure 20B). This result was interesting and suggested that Bδ is involved in the DNA damage response, a process activated by UV radiation, which repairs DNA mutations that can lead to genomic instability and cancer [260]. In support of this theory, a recent study demonstrated that DNA damage could inhibit Greatwall (GWL)/MASTL activity [261], a protein known to inhibit PP2A-Bδ complexes [120, 259]. This, is turn, would presumably increase Bδ activity, thus inhibiting cell cycle progression (DNA damage-induced arrest) to allow for DNA damage recovery. Once the damage has been repaired, GWL is released from inhibition to promote checkpoint recovery and reentry into the cell cycle. However, when Bδ cannot become activated by suppression of GWL as is the case for the Bδ knockdown cells in this study, the cells may still be able to enter mitosis thereby perpetuating the DNA damage which eventually may become so extensive that the cell can longer sustain itself and must undergo apoptosis. Indeed, this was observed in HeLa-Bδ knockdown cells, with cells undergoing abnormal

mitosis and eventually succumbing to apoptosis either within interphase or during mitosis.

In order to link the biological relevance of the finding that knocking down Bδ in HeLa cells results in apoptosis, HeLa-H2B-GFP cells were transfected with SVST to determine if expression of this T antigen also promotes cell death. Considering the hypothesis that SVST targets the methylation-sensitive subunit, Bδ, it was expected that similar mitotic defects would occur upon SVST expression. Cells were again visualized by timelapse microscopy to observe mitotic progression in cells that had been successfully transfected with either an empty control vector or SVST (red cells in Figure 21C). As anticipated from the Bδ findings, SVST expression also triggered mitotic defects within 3 days post-transfection, including lagging chromosomes during metaphase plate formation, which eventually resulted in apoptosis. These results suggest that SVST does indeed likely target Bδ subunit thereby supporting the hypothesis that SVST modulates PP2A function by replacing the methylation-sensitive B-type subunits. Furthermore, the finding that SVST induces apoptosis in HeLa cells is somewhat novel considering the fact that SVST expression has been generally linked in most studies to promoting the cell cycle and preventing apoptosis (reviewed in [172]), although two studies do confirm the issues of mitosis [262] or enhance apoptosis [263] in SVST-expressing cells. One can speculate that expression of SVST, or reduction of Bδ for that matter, may affect different cell types in distinctive manners. Therefore, considering the similarity of effect between Bδ knockdown and SVST expression in HeLa cells and the fact that SVST promotes transformation in HEKTERASB56γ cells, it is likely that knocking down Bδ in the HEKTERASB56γ would also promote transformation. This

potential disparity of effects would likely be due to the fact that HeLa and HEKTERASB56γ have different oncogenic pathways activated. Indeed, while both cell lines have dysfunctional p53 and pRb function [178, 264], the HEKTERASB56γ cells have additional oncogenic triggers including highly exogenously expressed hTERT and H-ras which may complement SVST expression in supporting cell cycle progression and survival.

Overall, the results from expressing the N-terminal portion of SVST or reducing methylation-sensitive B-type subunits in conjunction with B'γ subunit knockdown reveal that the mechanism by which MT and ST antigens promote transformation is rather complex. It appears that while reduction of methylation-sensitive PP2A complexes via knockdown of the PP2A methyltransferase, LCMT-1, can promote transformation, reducing methylation-sensitive B-type subunits, specifically Bα and B'α, separately does not confer this phenotype. Thus, it is possible that the T antigens use a strategy of simultaneously targeting a minimum number of PP2A B-type subunits required to overcome a variety of PP2A's antigrowth and antisurvival signaling to promote transformation. Furthermore, if any one of these methylation-sensitive PP2A complexes is not targeted, then robust transformation may not be achieved as was demonstrated in these experiments. In other words, reduction of only a subset of methylation-sensitive complexes may not be sufficient to mimic MT or ST-mediated transformation. It is also possible that these T antigens may modulate methylation directly by some unknown mechanism to specifically reduce assembly of methylation-sensitive complexes rather than just simply replacing the methylation-sensitive B-type subunits.

It was also demonstrated that the N-terminal portion of SVST does not on its own further enhance transformation. This result suggests that the role of the SVST J domain may be to support transformation only when functioning in the context of PP2A inhibition. It is likely that the chaperone activity of the N-terminal domain is important for assisting in PP2A modulation in cis or may even function in redirecting T antigen-targeted PP2A complexes to other substrates. In any case, this domain of SVST must remain intact with its C-terminal, PP2A-dependent domain in order to assist in promoting robust transformation. All in all, the results presented here thus far strongly suggest that multiple methylation-sensitive PP2A complexes are inhibited during transformation. Considering the fact that reducing PP2Ac methylation by LCMT-1 knockdown promotes transformation, this implies that increasing methylation may indeed be a possible strategy for anticancer therapies.

Sustained PP2Ac Methylation Enhances the Tumor Suppressive Function of PP2A through Down-regulation of the Akt Pathway

In Chapter 3.1, results demonstrated that reducing the assembly of PP2A complexes containing methylation-sensitive B-type subunits by knocking down LCMT-1 in HEKTERASB56γ cells enhanced cellular transformation in an Akt-dependent manner. Correspondingly, I hypothesized that DNA tumor virus MT and ST oncoproteins likely circumvent the tumor suppressive effects of PP2Ac methylation to exert their transforming effects. Overall, the findings support the theory that promoting PP2Ac methylation would likely reduce transformation in this system and, in fact, may represent a novel strategy for anticancer therapies. Therefore, to continue to explore this notion,

HEKTERASB56γ cells were knocked down for PME-1, the PP2Ac methylesterase, to enhance PP2Ac methylation. Reduction of PME-1 did, indeed, significantly enhance PP2Ac methylation and caused a significant reduction in transformation in this cell system. Furthermore, in agreement with the LCMT-1 knockdown findings, reducing PME-1 did not affect growth or Akt pathway activation in anchorage-dependent conditions. Only when PME-1 knockdown cells were subjected to anchorage-independent growth conditions, were significant alterations in pathway signaling observed, with significant decreases in the activating phosphorylations of Akt and of Akt substrates. Additionally, the activating phosphorylation on rpS6, which was affected in LCMT-1 knockdown cells, was also significantly reduced suggesting that the S6 kinase pathway is also being downregulated. Overall, these compiled results lead me to propose a model for MT and ST oncoprotein-mediated transformation in which methylation-insensitive viral B-type subunits (MT and ST) replace methylation-sensitive cellular PP2A B-type subunits to circumvent the antigrowth, antiproliferative effects of methylation-sensitive PP2A heterotrimers (Figure 25). Furthermore, increasing PP2Ac methylation by modulating LCMT-1 or PME-1 can directly regulate, though PP2A complex assembly, cell growth and survival and can itself support the tumor suppressive role of PP2A.

Over the past decade, many studies have focused on elucidating the role of PME-1 and its function in regulating PP2A. In early studies of PP2Ac methylation, it was determined that methylation is a highly regulated process which is both spatially and temporally controlled. Results have demonstrated that PP2Ac methylation levels change in a cell cycle-dependent manner [49, 50] and that LCMT-1 and PME-1 are more highly

expressed in the cytosol and nucleus, respectively [37]. These initial findings suggest that the cell precisely regulates PP2Ac methylation as a means to control PP2A function and specificity. It is well established that PP2Ac methylation does not alter PP2A function by altering its catalytic activity but rather regulates PP2A complex formation with the B-type subunits [37, 42-44, 265]. While the role of LCMT-1 in this process is well documented, the involvement of PME-1 has only recently become the focus of much scrutiny. PME-1 has recently been discovered to not only function as a methylesterase for PP2Ac, but to also serve as a regulator of PP2A biogenesis (reviewed in [24]). Recently, it was determined that PME-1 can associate with an inactive pool of PP2A A/C dimers [41] which may represent up to 25% of the total PP2A pool in the cell [266]. PME-1 stabilizes this inactive pool of dimers protecting them from disassembly and degradation until the cell requires additional active PP2A. Once the cell signals for new PP2A assembly, PME-1-bound PP2A is reactivated by PTPA, a prolyl peptidyl isomerase specific for PP2Ac, which then allows for PP2Ac methylation. This process appears to be important for PP2A biogenesis, considering the fact that newly translated PP2Ac is inactive and requires stabilization prior to assembly into complexes [13, 236].

The findings here strongly indicate that supporting PP2Ac methylation by reducing PME-1 reduces transformation through suppressing progrowth, prosurvival signaling as demonstrated by reduced Akt signaling and rpS6 phosphorylation. Therefore, one can speculate that enhancing PP2Ac methylation promotes PP2A's tumor suppressive role by favoring the assembly of methylation-sensitive B-type subunits into PP2A holoenzymes, complexes known to be involved in the dephosphorylation and inactivation of multiple progrowth/prosurvival pathways and processes. In support of this

theory, one study found that knocking down PME-1 in HeLa cells also resulted in reduced anchorage-independent growth in soft agar and decreased ERK/MAPK activity. Furthermore, the same study found that PME-1 expression strongly correlated with progression of low grade gliomas to malignant glioblastomas [238], thereby emphasizing the biological relevance of their findings and my findings. Overall, knocking down PME-1 reduces transformation demonstrating that enhancing PP2Ac methylation, and thus the assembly of PP2A holoenzymes containing methylation-sensitive B-type subunits, is essential for PP2A's tumor suppressive role. Furthermore, it appears from this study that reducing PME-1 only alters the growth and signaling of anchorage-independent cells, thus indicating that the effects of targeting PME-1 may be fairly specific for cancer-like cells. Therefore, modulation of PP2Ac methylation may prove in time to be beneficial as a therapy against some cancer types.

Closing Remarks: Implications for Cancer

The pathways found to be impacted in this study, by altering PP2Ac methylation levels via modulation of LCMT-1 and PME-1 expression, are highly relevant to human cancer. Akt activation is common in cancer and, in many cases, correlates with a poor prognosis and resistance to current therapies [267]. As a direct target and important effector of mTOR in the control of cell growth and proliferation, p70 S6K has also been implicated in tumorigenesis [225, 268]. Increases in the amount and/or activation of p70 S6K have been reported in multiple cancers or cancer cell lines [269-273]. Therefore, the growth and survival pathways modulated by PP2Ac methylation are important not only for SV40- and polyomavirus-mediated transformation but also for human cancer. This

study also demonstrates that modulation of PP2Ac methylation is sufficiently potent to regulate these growth and survival pathways and that LCMT-1 is important for the tumor-suppressive function of PP2A. Furthermore, it appears that while reduction of the methylation-sensitive B'γ in HEKTER cells is sufficient for promoting transformation, knocking down additional methylation-sensitive B-type subunits, subunits targeted by MT and ST, in conjunction with B'γ does not further enhance transformation even though reducing LCMT-1 does contribute to transformation. This suggests a model where the transforming capacity of these T antigens is due to the fact that they replace multiple methylation-sensitive B-type subunits simultaneously and, therefore, use this strategy to impede a variety of progrowth/prosurvival pathways in this cell system (Figure 26). Of note, knockdown of LCMT-1 in MCF7 breast cancer cells resulted in enhanced anchorage-independent growth in 3 of 5 assays whereas reduction of LCMT-1 in LNCaP (prostate cancer) and H1299 (lung cancer) cells had variable effects (data not shown). These findings strongly suggest that the effects of reducing PP2Ac methylation are cell type specific and are likely dependent on the oncogenic signals already present in the cell. Therefore, increasing PP2Ac methylation levels may have anticancer effects on cells that have similar oncogenic triggers as HEKTERASB56 cells.

The finding that suppression of LCMT-1 resulted in enhanced anchorage-independent growth provided important insights that justified the investigation into the effects of targeting PME-1. Indeed, the enhanced PP2Ac methylation resulting from PME-1 knockdown reduced anchorage-independent growth, and thus transformation, by affecting Akt pathway activation in the HEKTERASB56γ cell system, thereby providing further support to the claim that PP2Ac methylation is tumor suppressive (Figure 25).

Although only Akt activation and rpS6 phosphorylation were measured as a readout for reduced transformation, it is likely that additional progrowth/prosurvival processes and pathways negatively regulated by PP2A complexes containing methylation-sensitive B-type subunits are being downregulated as well. One can speculate that many of these processes and pathways that are being downregulated due to the reduction of PME-1, and thus the activation of methylation-sensitive PP2A complexes, represent mechanisms that are upregulated in cancer. Considering that the methylation-sensitive PP2A complexes can counteract multiple processes which contribute to several hallmarks of cancer include sustained proliferative signaling, resistance to cell death, and ability to grow without a substratum, drug compounds that can support these complexes may have potential in cancer therapy. Furthermore, on the basis of these findings, it seems likely that down-regulation of LCMT-1 amount or activity will be found to contribute to the development of human cancers and that compounds that increase PP2A methylation (e.g., PME-1 inhibitors) will exhibit anticancer activity. In fact, one group has described a class of inhibitors, aza-β-lactam (ABL) compounds, which can selectively target PME-1 and inhibit its methylesterase activity in both human and mouse cell models [274]. Utilizing this class of inhibitors, a great wealth of knowledge will be acquired as to the role of PME-1 in PP2A function and to the biological relevance of suppressing PME-1. The attractive possibility that the pharmacological effects of these PME-1 inhibitors can result in reduced tumor growth and/or survival is quite compelling and is certainly supported by the findings presented herein.

Future Directions

Overall, these results have laid the groundwork for the further study of how DNA tumor virus MT and ST oncoproteins promote transformation by circumventing cellular control of PP2A by methylation. It would be interesting to determine if MT and ST are able to downregulate PP2A methylation directly, thereby allowing for efficient replacement of the methylation-sensitive B-type subunits (Figure 26). This would represent a novel strategy by which DNA tumor viruses override the negative regulatory roles, and thus tumor suppressive function, of PP2A. Furthermore, examining the effects of simultaneously knocking down the methylation-sensitive B-type subunits used in this study in HEKTERASB56γ cells would presumably further support the model that MT and ST promote robust transformation through replacement of these subunits. In all likelihood, these cells would more fully mimic the level of transformation (measured by colony number and size) observed when LCMT-1 is knocked down or when SVST is expressed due to the synergistic effects of reducing all PP2A complexes containing methylation-sensitive B-type subunits. With regards to the finding that knocking down Bδ in HeLa cells induces apoptosis, it may be useful to test for these effects in other cancer lines that have similar oncogenic triggers as the HeLa cells. Additionally, it would be interesting to explore the role of Bδ in DNA damage-induced arrest considering the fact that DNA damage causes inhibition of GWL, an upstream inhibitor of Bδ. Regarding the finding that suppressing B'α reduced transformation in HEKTERASB56γ cells, it is tempting to speculate that knocking down B'α in cancer lines expressing low or aberrant B'γ (e.g. lung cancer lines [171]) could possibly reduce anchorage-independent growth and therefore, may have therapeutic potential. Finally, in the future, it will be intriguing

to test the possible anti-transformative effects of the newly discovered PME-1 inhibitors (ABL) which has great potential, as implied by the current research here, as a novel therapy for cancer.

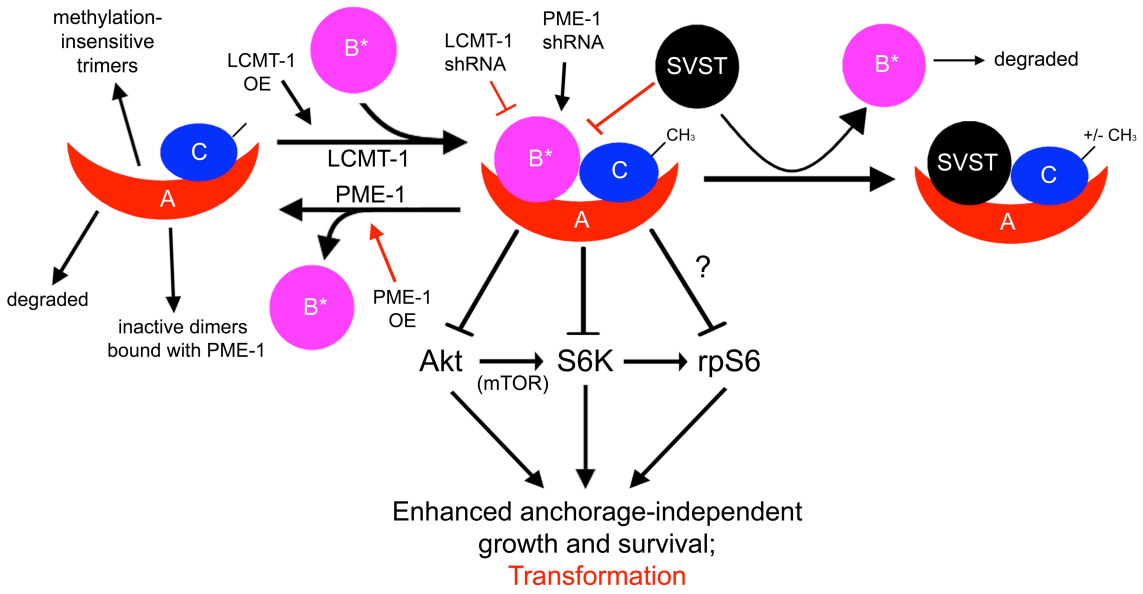


Figure 25. Proposed model for the negative regulation of anchorage-independent growth and survival by methylation-sensitive PP2A heterotrimers: the effects of expressing SV40 small T (SVST) or modulating PP2Ac methylation on transformation. Methylation of PP2Ac through LCMT-1 promotes incorporation of methylation-sensitive B-type subunits (B*) into heterotrimers that dephosphorylate and thereby inactivate Akt and S6K. PP2A methylation-sensitive heterotrimers can act on S6K in both an Akt-dependent and independent manner to block downstream survival and translation signals and thus transformation. PP2A methylation-sensitive heterotrimers also promote the dephosphorylation of rpS6 independently of S6K to block translation, although it is not known if this is a direct or indirect effect. Methylation-insensitive SVST (or PyST and PyMT) oncoprotein replaces methylation-sensitive B-type subunits, promoting transformation by preventing the dephosphorylation and inactivation of Akt and S6K. Transformation is also enhanced by reducing methylation-sensitive PP2A heterotrimers by inhibition of LCMT-1 (e.g., through shRNA or mutation) or PME-1 overexpression

(PME-OE). Conversely, transformation is reduced by supporting PP2Ac methylation and thus methylation-sensitive PP2A complexes by inhibition of PME-1 (e.g., through shRNA or mutation) or LCMT-1 overexpression (LCMT-1 OE). Red arrows and block lines signify protransformation effects. For simplicity, only the targets of methylation-sensitive PP2A heterotrimers examined in this study are shown in this schematic. Figure and legend adjusted from [220].

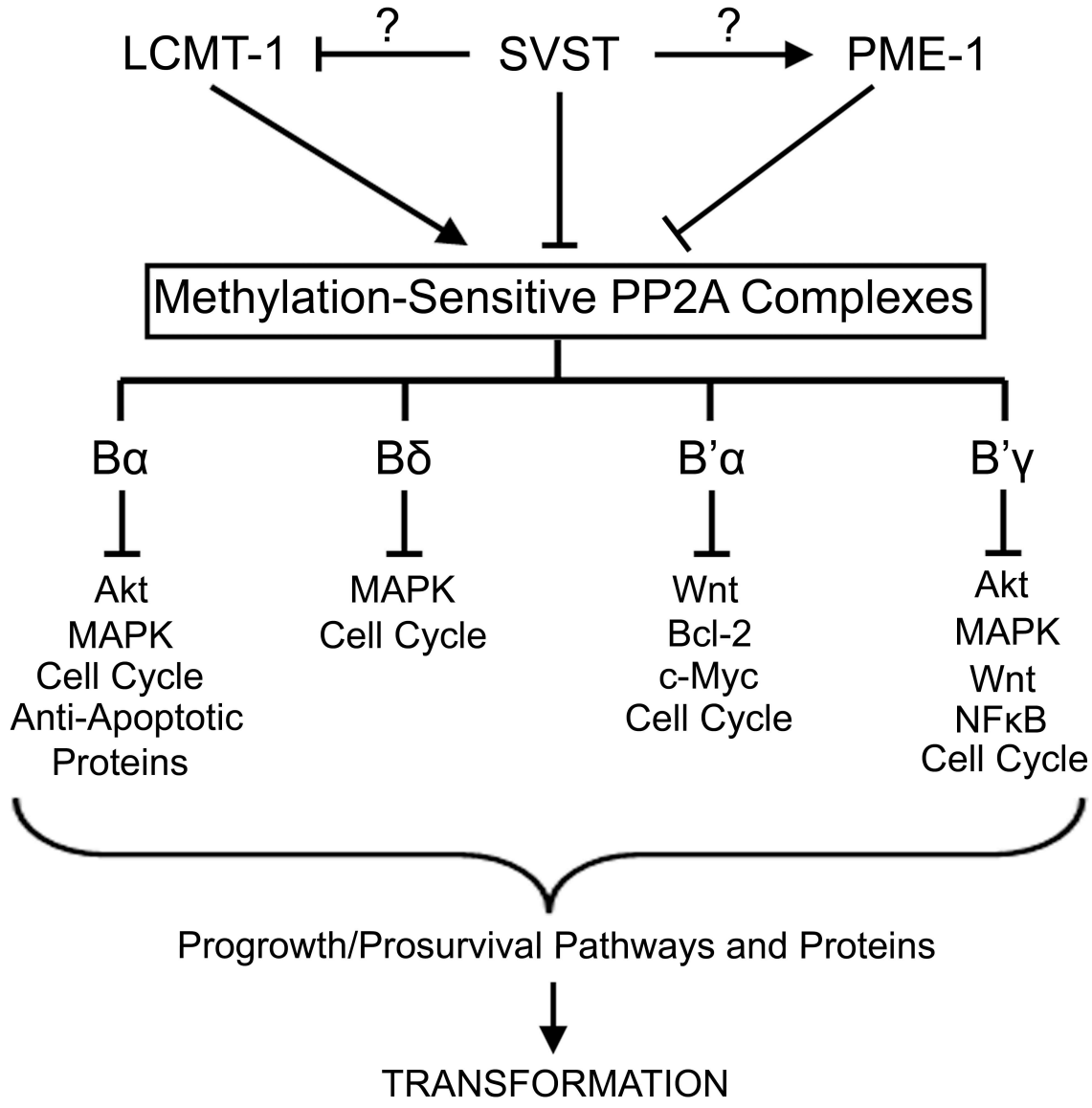


Figure 26. Proposed model for the strategies exploited by SV40 small T (SVST) to promote transformation. Research strongly supports the mechanism that SVST drives transformation through the upregulation of several key progrowth/prosurvival pathways and processes, including cell cycle progression and antiapoptotic machinery. Findings here and elsewhere suggest that SVST activates these processes through the downregulation of a specific subset of PP2A complexes, heterotrimers that are dependent

on PP2Ac methylation, which are broadly involved in the regulation of antigrowth/antisurvival mechanisms. While knockdown of B'γ alone can partially mimic SVST-mediated transformation and reduction of LCMT-1 further enhances transformation in HEKTERASB56γ cells, reduction of additional methylation-sensitive B-type subunits in conjunction with B'γ knockdown cannot. This suggests that SVST probably concomitantly inhibits most, if not all, methylation-sensitive B-type subunits to produce a synergistic effect to enhance transformation. Considering the fact that most of the methylation-sensitive B-type subunits share functions (e.g. cell cycle regulation), it appears necessary for SVST to inhibit all of these methylation-sensitive complexes simultaneously in order to override their antigrowth/antisurvival signals. Thus, SVST, and likely PyMT and PyST, utilizes a strategy of circumventing cellular control of PP2A by methylation to promote transformation thereby blocking antigrowth/antisurvival signaling. The model here also takes into account the possibility that these T antigens may modulate methylation directly by modulating LCMT-1 and/or PME-1 function, rather than just simply replacing the methylation-sensitive B-type subunits, thereby allowing for efficient replacement of the methylation-sensitive B-type subunits.

CHAPTER 5

References

1. McConnell, J.L. and B.E. Wadzinski, Targeting protein serine/threonine phosphatases for drug development. *Mol Pharmacol* 2009. **75**: p. 1249-61.
2. Honkanen, R.E. and T. Golden, Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? *Curr Med Chem*, 2002. **9**(22): p. 2055-75.
3. Hernandez, M., A. Lachmann, S. Zhao, K. Xiao and A. Ma'ayan, Inferring the sign of kinase-subbstrate interactions by combinging quantitative phosphoproteomics with a literature-based mammalian kinome network. *BIBE*, 2010: p. 180-4.
4. Janssens, V., J. Goris and C. Van Hoof, PP2A: the expected tumor suppressor. *Curr Opin Genet Dev*, 2005. **15**(1): p. 34-41.
5. Guergnon, J., A.N. Godet, A. Galioot, P.B. Falanga, J.H. Colle, X. Cayla, et al., PP2A targeting by viral proteins: A widespread biological strategy from DNA/RNA tumor viruses to HIV-1. *Biochim Biophys Acta*, 2011. **1812**: p. 1498-1507.
6. Cheng, X., Hart, G.W., Alternative O-glycosylation/O-phosphorylation of serine-16 in murine estrogen receptor beta: post-translational regulation of turnover and transactivation activity. *J Biol Chem*, 2001. **276**(13): p. 10570-10575.

7. Mukherjee, S., Keitany, G., Li, Y., Wang, Y., Ball, H.L., Goldsmith, E.J., Orth, K., Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. *Science*, 2006. **312**: p. 1211-1214.

8. Moorhead, G.B.G., L. Trinkle-Mulcahy and A. Ulke-Lemee, Emerging roles of nuclear protein phosphatases. *Mol Cell Bio*, 2007. **8**: p. 234-44.

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

10. Bodenmiller, B., S. Wanka, C. Kraft, J. Urban, D. Campbell, P.G. Pedrioli, et al., Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. *Sci Signal*, 2010. **3**(153): p. rs4.

11. Manning, G., D.B. Whyte, R. Martinez, T. Hunter and S. Sudarsanam, The protein kinase compliment of the human genome. *Science*, 2002. **298**: p. 1912-34.

12. Alonso, A., J. Sasin, N. Bottini, I. Friedberg, I. Friedberg, A. Osterman, et al., Protein tyrosine phosphatases in the human genome. *Cell*, 2004. **117**: p. 699-711.

13. Fellner, T., D.H. Lackner, H. Hombauer, P. Piribauer, I. Mudrak, K. Zaragoza, et al., A novel and essential mechanism determining specificity and activity of protein phosphatase 2A (PP2A) in vivo. *Genes Dev*, 2003. **17**(17): p. 2138-50.

14. Sontag, E., Protein phosphatase 2A: the trojan horse of cellular signaling. *Cell Signal*, 2001. **13**: p. 7-16.

15. Virshup, D.M., Protein phosphatase 2A: a panoply of enzymes *Curr Opin Cell Biol*, 2000. **12**(2): p. 180-5.

16. Martin, M., R. Kettmann and F. Dequiedt, Recent insights into Protein Phosphatase 2A structure and regulation: the reasons why PP2A is no longer considered as a lazy passive housekeeping enzyme. *Biotechnol Agron Soc Environ*, 2010. **14**(1): p. 243-252.

17. Mumby, M., PP2A: Unveiling a reluctant tumor suppressor. *Cell*, 2007. **130**: p. 21-24.

18. Murata, K., J. Wu and D.L. Brautigan, B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. *Proc Natl Acad Sci U S A*, 1997. **94**(20): p. 10624-9.

19. Westermarck, J. and W.C. Hahn, Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med*, 2008. **14**(4): p. 152-60.

20. Hemmings, B.A., C. Adams-Pearson, F. Maurer, P. Muller, J. Goris, W. Merlevede, et al., alpha- and beta-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry*, 1990. **29**: p. 3166-3173.

21. Gotz, J., A. Probst, E. Ehler, B. Hemmings and W. Kues, Delayed embryonic lethality in mice lacking protein phosphatase 2A catalytic subunit Calpha. *Proc Natl Acad Sci U S A*, 1998. **95**(21): p. 12370-5.

22. Schonthal, A.H., Role of serine/threonine protein phosphatase 2A in cancer. *Cancer Lett*, 2001. **170**(1): p. 1-13.

23. Janssens, V. and J. Goris, Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J*, 2001. **353**(Pt 3): p. 417-39.

24. Haesen, D., W. Sents, E. Ivanova, C. Lambrecht and V. Janssens, Cellular inhibitors of protein phosphatase PP2A in cancer. *Biomed Res*, 2012. **23**: p. SI 197-211.

25. Millward, T.A., S. Zolnierowicz and B.A. Hemmings, Regulation of protein kinase cascades by protein phosphatase 2A. [Review] [89 refs]. *Trends Biochem Sci*, 1999. **24**(5): p. 186-91.

26. Xu, Y., Y. Xing, Y. Chen, Y. Chao, Z. Lin, E. Fan, et al., Structure of the protein phosphatase 2A holoenzyme. *Cell*, 2006. **127**: p. 1239-51.

27. Xie, H. and S. Clarke, Methyl esterification of C-terminal leucine residues in cytosolic 36-kDa polypeptides of bovine brain. A novel eucaryotic protein carboxyl methylation reaction. *J Biol Chem*, 1993. **268**(18): p. 13364-71.

28. Lee, J. and J. Stock, Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase. *J Biol Chem*, 1993. **268**(26): p. 19192-5.

29. Xie, H. and S. Clarke, Protein phosphatase 2A is reversibly modified by methyl esterification at its C-terminal leucine residue in bovine brain. *J Biol Chem*, 1994. **269**(3): p. 1981-4.

30. Favre, B., S. Zolnierowicz, P. Turowski and B.A. Hemmings, The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo. *J Biol Chem*, 1994. **269**(23): p. 16311-7.

31. Lee, J.A. and D.C. Pallas, Leucine carboxylmethyltransferase-1 (LCMT-1). *AfCS-Nature Molecule Pages*, 2006. **A003167**.

32. De Baere, I., R. Derua, V. Janssens, C. Van Hoof, E. Waelkens, W. Merlevede, et al., Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. *Biochemistry*, 1999. **38**(50): p. 16539-47.

33. Leulliot, N., S. Quevillon-Cheruel, I. Sorel, I.L. de La Sierra-Gallay, B. Collinet, M. Graille, et al., Structure of protein phosphatase methyltransferase 1 (PPM1), a leucine carboxyl methyltransferase involved in the regulation of protein phosphatase 2A activity. *J Biol Chem*, 2004. **279**(9): p. 8351-8.

34. Lee, J., Y. Chen, T. Tolstykh and J. Stock, A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain. *Proc Natl Acad Sci U S A*, 1996. **93**(12): p. 6043-7.

35. Ogris, E., X. Du, K.C. Nelson, E.K. Mak, X.X. Yu, W.S. Lane, et al., A protein phosphatase methylesterase (PME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A. *J Biol Chem*, 1999. **274**(20): p. 14382-91.

36. Xie, H. and S. Clarke, An enzymatic activity in bovine brain that catalyzes the reversal of the C-terminal methyl esterification of protein phosphatase 2A. *Biochem Biophys Res Commun*, 1994. **203**(3): p. 1710-5.

37. Longin, S., K. Zwaenepoel, E. Martens, J.V. Louis, E. Rondelez, J. Goris, et al., Spatial control of protein phosphatase 2A (de)methylation. *Exp Cell Res*, 2007.

38. Kloeker, S., J.C. Bryant, S. Strack, R.J. Colbran and B.E. Wadzinski, Carboxymethylation of nuclear protein serine/threonine phosphatase X. *Biochem J*, 1997. **327**(Pt 2): p. 481-6.

39. Longin, S. and J. Goris, *Reversible methylation of protein phosphatase 2A*, in *The Enzymes, Protein Methyltransferase*, S.G. Clarke and F. Tamanoi, Editors. 2006, Elsevier/Academic Press: Oxford, UK. p. 303-324.

40. Lee, J.A. and D.C. Pallas, Protein phosphatase methylesterase-1 (PME-1). *AfCS-Nature Molecule Pages*, 2006. **A003170**.

41. Longin, S., J. Jordens, E. Martens, I. Stevens, V. Janssens, E. Rondelez, et al., An inactive protein phosphatase 2A population is associated with methylesterase and can be reactivated by the phosphotyrosyl phosphatase activator. *Biochem J*, 2004. **Pt**.

42. Ogris, E., D.M. Gibson and D.C. Pallas, Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not polyomavirus middle tumor antigen. *Oncogene*, 1997. **15**(8): p. 911-7.

43. Wei, H., D.G. Ashby, C.S. Moreno, E. Ogris, F.M. Yeong, A.H. Corbett, et al., Carboxymethylation of the PP2A catalytic subunit in Saccharomyces cerevisiae is required for efficient interaction with the B-type subunits Cdc55p and Rts1p. *J Biol Chem*, 2001. **276**(2): p. 1570-7.

44. Yu, X.X., X. Du, C.S. Moreno, R.E. Green, E. Ogris, Q. Feng, et al., Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen. *Mol Biol Cell*, 2001. **12**(1): p. 185-99.

45. Wu, J., T. Tolstykh, J. Lee, K. Boyd, J.B. Stock and J.R. Broach, Carboxyl methylation of the phosphoprotein phosphatase 2A catalytic subunit promotes its

functional association with regulatory subunits in vivo. *Embo J*, 2000. **19**(21): p. 5672-5681.

46. Tolstykh, T., J. Lee, S. Vafai and J.B. Stock, Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits. *Embo J*, 2000. **19**(21): p. 5682-5691.

47. Evans, D.R. and B.A. Hemmings, Mutation of the C-terminal leucine residue of PP2Ac inhibits PR55/B subunit binding and confers supersensitivity to microtubule destabilization in Saccharomyces cerevisiae. *Mol Gen Genet*, 2000. **264**(4): p. 425-32.

48. Gentry, M.S., Y. Li, H. Wei, F.F. Syed, S.H. Patel, R.L. Hallberg, et al., A novel assay for protein phosphatase 2A (PP2A) complexes in vivo reveals differential effects of covalent modifications on different Saccharomyces cerevisiae PP2A heterotrimers. *Eukaryot Cell*, 2005. **4**(6): p. 1029-40.

49. Turowski, P., A. Fernandez, B. Favre, N.J. Lamb and B.A. Hemmings, Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression. *J Cell Biol*, 1995. **129**(2): p. 397-410.

50. Zhu, T., S. Matsuzawa, Y. Mizuno, C. Kamibayashi, M.C. Mumby, N. Andjelkovic, et al., The interconversion of protein phosphatase 2A between PP2A1 and PP2A0 during retinoic acid-induced granulocytic differentiation and a modification on the catalytic subunit in S phase of HL-60 cells. *Arch Biochem Biophys*, 1997. **339**(1): p. 210-7.

51. Bryant, J.C., R.S. Westphal and B.E. Wadzinski, Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit. *Biochem J*, 1999. **339**(Pt 2): p. 241-6.

52. Longin, S., K. Zwaenepoel, J.V. Louis, S. Dilworth, J. Goris and V. Janssens, Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit. *J Biol Chem*, 2007. **282**(37): p. 26971-80.

53. Lee, J.A. and D.C. Pallas, Leucine carboxyl methyltransferase-1 is necessary for normal progression through mitosis in mammalian cells. *J Biol Chem*, 2007. **282**: p. 30974-30984.

54. Stanevich, V., L. Jiang, K.A. Satyshur, Y. Li, P.D. Jeffrey, Z. Li, et al., The structural basis for tight control of PP2A methylation and function by LCMT-1. *Mol Cell*, 2011. **41**(3): p. 331-342.

55. Vafai, S.B. and J.B. Stock, Protein phosphatase 2A methylation: a link between elevated plasma homocysteine and Alzheimer's Disease. *FEBS Lett*, 2002. **518**(1-3): p. 1-4.

56. Sontag, E., C. Hladik, L. Montgomery, A. Luangpirom, I. Mudrak, E. Ogris, et al., Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis. *J Neuropathol Exp Neurol*, 2004. **63**(10): p. 1080-91.

57. Sontag, E., V. Nunbhakdi-Craig, J.M. Sontag, R. Diaz-Arrastia, E. Ogris, S. Dayal, et al., Protein phosphatase 2A methyltransferase links homocysteine metabolism with tau and amyloid precursor protein regulation. *J Neurosci*, 2007. **27**(11): p. 2751-9.

58. Sun, H.a.W., Y., Novel Ser/Thr protein phosphatases in cell death regulation. *Physiol*, 2012. **27**: p. 43-52.

59. Song, G., Ouyang, G., Bao, S., The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*, 2005. **9**(1): p. 59-71.

60. Manning, B.D.a.C., L.C., AKT/PKB signaling: Navigating downstream. *Cell*, 2007. **129**: p. 1261-1274.

61. Kuo, Y.C., K.Y. Huang, C.H. Yang, Y.S. Yang, W.Y. Lee and C.W. Chiang, 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*, 2008. **283**(4): p. 1882-92.

62. Andrabi, S., O.V. Gjoerup, J.A. Kean, T.M. Roberts and B. Schaffhausen, Protein phosphatase 2A regulates life and death decisions via Akt in a context-dependent manner. *Proc Natl Acad Sci U S A*, 2007. **104**(48): p. 19011-6.

63. Hanada, M., Feng, J., Hemmings, B.A, Structure, regulation and function of PKB/AKT—a major therapeutic target. *Biochim Biophys Acta*, 2004. **1697**: p. 3-16.

64. Liao, Y.a.H., M.C., Physiological regulation of Akt activity and stability. *Am J Trans Res*, 2010. **2**(1): p. 19-42.

65. Ugi, S., T. Imamura, H. Maegawa, K. Egawa, T. Yoshizaki, K. Shi, et al., Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1 adipocytes. *Mol Cell Biol*, 2004. **24**(19): p. 8778-89.

66. Trotman, L.C., Alimonti, A., Scaglioni, P.P., Koutcher, J.A., Cordon-Cardo, C., Pandolfi, P.P., Identification of a tumour suppressor network opposing nuclear Akt function. *Nature*, 2006. **441**: p. 523-527.

67. Pim, D., P. Massimi, S.M. Dilworth and L. Banks, Activation of the protein kinase B pathway by the HPV-16 E7 oncoprotein occurs through a mechanism involving interaction with PP2A. *Oncogene*, 2005.

68. Vereshchagina, N., Ramel, M.C., Bitoun, E., Wilson, C., The protein phosphatase PP2A-B' subunit Widerborst is a negative regulator of cytoplasmic activated Akt and lipid metabolism in Drosophila. *J Cell Sci*, 2008. **121**: p. 3383-3392.

69. Van Kanegan, M.J.A., D.G., Wadzinski, B.E., Strack, S., Distinct protein phosphatase 2A heterotrimers modulate growth factor signaling to extracellular signal-regulated kinase and Akt. *J Biol Chem*, 2005. **280**(43): p. 36029-36.

70. Ruvolo, P.P., Ceramide regulates cellular homeostasis via diverse stress signaling pathways. *Leukemia*, 2001. **15**: p. 1153-1160.

71. Dobrowsky, R.T., C. Kamibayashi, M.C. Mumby and Y.A. Hannun, Ceramide activates heterotrimeric protein phosphatase 2A. *J Biol. Chem.*, 1993. **268**(21): p. 15523-30.

72. Salinas, M., Lopez-Valdaliso, R., Martin, D., Alvarez, A., and Cuadrado, and A., Inhibition ofPKB/Akt1 by C2-ceramide involves activation of ceramide-activated protein phosphatase in PC12 cells. *Neurosci*, 2000. **15**: p. 156-169.

73. Rocher, G., C. Letourneux, P. Lenormand and F. Porteu, Inhibition of B56-containing protein phosphatase 2As by the early response gene IEX-1 leads to control of Akt activity. *J Biol Chem*, 2007. **282**(8): p. 5468-77.

74. Padmanabhan, S., Mukhopadhyay, A., Narasimhan, S.D., Tesz, G., Czech, M.P., Tissenbaum, H.A., A PP2A regulatory subunit PPTR-1 regulates the C. elegans Insulin/IGF-1 signaling pathway by modulating AKT-1 phosphorylation. *Cell*, 2009. **136**: p. 939-951.

75. Ruvolo, P.P., Qui, Y.H., Coombes, K.R., Zhang, N., Ruvolo, V.R., Borthakur, G., Konopleva, M., Andreeff, M., Kornblau, S.M., Low expression of PP2A regulatory subunit B55α is associated with T308 phosphorylation of AKT and shorter complete remission duration in acute myeloid leukemia patients. *Leukemia*, 2011. **25**(11): p. 1711-1717.

76. Crowell, J.A., Steele, V.E., Fay, J.R., Targeting the AKT protein kinase for cancer chemoprevention. *Mol Cancer Ther*, 2007. **6**: p. 2139-2148.

77. Jope, R.S.a.J., G.V.W., The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci*, 2004. **29**(2): p. 95-102.

78. Cross, D.A., D.R. Alessi, P. Cohen, M. Andjelkovich and B.A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, 1995. **378**(6559): p. 785-9.

79. Fukumoto, S., Hsieh, C.M., Maemura, K., Layne, M.D., Yet, S.F., Lee, K.H., Matsui, T., Rosenzweig, A., Taylor, W.G., Rubin, J.S., Perrella, M.A., Lee, M.E., Akt Participation in the Wnt Signaling Pathway through Dishevelled. *J Biol Chem*, 2001. **276**(20): p. 17479-17483.

80. Arnold, H.K., Sears, R.C., A tumor suppressor role for PP2A-B56alpha through negative regulation of c-Myc and other key oncoproteins. *Cancer Metastasis Rev* 2008. **27**(2): p. 147-158.

81. Li, X., H.J. Yost, D.M. Virshup and J.M. Seeling, Protein phosphatase 2A and its B56 regulatory subunit inhibit Wnt signaling in Xenopus. *Embo J*, 2001. **20**(15): p. 4122-31.

82. Seeling, J.M., J.R. Miller, R. Gil, R.T. Moon, R. White, and D. M. Virshup, Regulation of β-Catenin signaling by the B56 subunit of Protein Phosphatase 2A. *Science*, 1999. **283**(5410): p. 2089-2091.

83. Sablina, A.A.H., M.; Colpaert, N.; Hahn, W.C., Identification of PP2A Complexes and Pathways Involved in Cell Transformation. *Cancer Research*, 2010. **70**: p. 10474-84.

84. Zhang, W., Yang, J., Liu, Y., Chen, X., Yu, T., Jia, J., and Liu, C., PR55alpha, a Regulatory Subunit of PP2A, Specifically Regulates PP2A-mediated Beta-Catenin Dephosphorylation. *J Biol Chem*, 2009. **284**(34): p. 22649–22656.

85. Hay, N. and N. Sonenberg, Upstream and downstream of mTOR (review). *Gene Dev*, 2004. **18**: p. 1926-45.

86. Laplante, M., Sabatini, D.M., mTOR signaling at a glance. *J Cell Sci*, 2009. **122**: p. 3589-3594.

87. Magnuson, B., Ekim, B., Fingar, D.C., Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signaling networks. *Biochem J*, 2012. **441**: p. 1-21.

88. Harris, T.E., Lawrence Jr., J.C., TOR signaling. *Sci STKE*, 2003. **212**: p. re15.

89. Fenton, T.R., Gout, I.T., Functions and regulation of the 70 kDa ribosomal S6 kinases. *Int J Biochem Cell Biol*, 2011. **43**: p. 47-59.

90. Inoki, K., H. Ouyang, Y. Li and K.L. Guan, Signaling by target of rapamycin proteins in cell growth control. *Microbiol Mol Biol Rev*, 2005. **69**(1): p. 79-100.

91. Peterson, R.T., B.N. Desai, J.S. Hardwick and S.L. Schreiber, Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. *Proc Natl Acad Sci U S A*, 1999. **96**(8): p. 4438-42.

92. Westphal, R.S., R.L. Coffee, Jr., A. Marotta, S.L. Pelech and B.E. Wadzinski, Identification of kinase-phosphatase signaling modules composed of p70 S6 kinase-protein phosphatase 2A (PP2A) and p21-activated kinase-PP2A. *J Biol Chem*, 1999. **274**(2): p. 687-92.

93. Hahn, K., Miranda, M., Francis, V.A., Vendrell, J., Zorzano, A., Teleman, A.A., PP2A regulatory subunit PP2A-B' counteracts S6K phosphorylation. *Cell Metab*, 2010. **11**: p. 438-444.

94. Petritsch, C., H. Beug, A. Balmain and M. Oft, TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest [In Process Citation]. *Genes Dev*, 2000. **14**(24): p. 3093-101.

95. Junttila, M.R., S.P. Li and J. Westermarck, Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation fo cell survival. *J FASEB*, 2008. **22**: p. 954-65.

96. Roberts, P.J.a.D., C.J., Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene*, 2007. **26**: p. 3291-3310.

97. Adams, D.G., R.L.C. Jr, H. Zhang, S. Pelech, S. Strack and B.E. Wadzinski, Positive regulation of Raf1-MEK1/2-ERK1/2 signaling by protein

serine/threonine phosphatase 2A holoenzymes. *J Biol Chem.*, 2005. **280**(52): p. 42644-54.

98. Sontag, E., S. Fedorov, C. Kamibayashi, D. Robbins, M. Cobb and M. Mumby, The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. *Cell*, 1993. **75**(5): p. 887-97.

99. Westermarck, J., T. Holmstrom, M. Ahonen, J.E. Eriksson and V.M. Kahari, Enhancement of fibroblast collagenase-1 (MMP-1) gene expression by tumor promoter okadaic acid is mediated by stress-activated protein kinases Jun N-terminal kinase and p38. *Matrix Biol*, 1998. **17**(8-9): p. 547-57.

100. Silverstein, A.M., C.A. Barrow, A.J. Davis and M.C. Mumby, Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. *Proc Natl Acad Sci U S A*, 2002. **99**(7): p. 4221-6.

101. Letourneux, C., G. Rocher and F. Porteu, B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *Embo J*, 2006.

102. Jiang, Y., Regulation of the cell cycle by protein phosphatase 2A in Saccharomyces cerevisiae *Microbiol Mol Biol Rev*, 2006. **70**(2): p. 440-449.

103. Burke, D.J., Interpreting spatial information and regulating mitosis in response to spindle orientation. *Gene Dev*, 2009. **23**: p. 1613-1618.

104. Domingo-Sananes, M.R., Kapuy, O., Hunt, T., Novak, B., Switches and latches: a biochemical tug-of-war between the kinases and phosphatases that control mitosis. *Phil Trans R Soc B*, 2011. **366**: p. 3584-3594.

105. Forester, C.M., Maddox, J., Louis, J.V., Goris, J., Virshup, D.M., Control of mitotic exit by PP2A regulation of Cdc25c and Cdk1. *PNAS*, 2007. **104**(50): p. 19867-19872.

106. Kawabe, T., G2 checkpoint abrogators as anticancer drugs. *Mol Cancer Ther*, 2004. **3**(4): p. 513-9.

107. Barr, F.A., Elliot, P.R. Gruneberg, U., Protein phosphatases and the regulation of mitosis. *J Cell Sci*, 2011. **124**(14): p. 2323-2334.

108. Wurzenberger, C.G., D.W., Phosphatases: providing safe passage through mitotic exit. *Mol Cell Biol*, 2011. **12**: p. 469-482.

109. Jang, Y.J., Ji, J.H., Choi, Y.C., Rye, C.J., Ko, S.Y., Regulation of Polo-like kinase 1 by DNA damage in mitosis. *J Biol Chem*, 2007. **282**(4): p. 2473-2482.

110. Lee, T.Y., Lai, T.Y., Lin, S.C., Wu, C.W., Ni, I.F., Yang, Y.S., Hung, L.Y., Law, B.K., Chiang, C.W., The B56gamma3 regulatory subunit of protein phosphatase 2A (PP2A) regulates S phase-specific nuclear accumulation of PP2A and the G1 to S transition. *J Biol Chem*, 2010. **285**(28): p. 21567-21580.

111. Jayadeva, G., Kurimchak, A., Garriga, J., Sotillo, E., Davis, A.J., Haines, D.S., Mumby, M., Grana, X., B55alpha PP2A holoenzymes modulate the phosphorylation status of the retinoblastoma-related protein p170 and its activation. *J Biol Chem*, 2010. **285**(39): p. 29863-29873.

112. Virshup, D.M., Kaldis, P., Enforcing the Greatwall in mitosis. *Science*, 2010. **330**: p. 1638-1639.

113. Margolis, S.S., Perry, J.A, Forester, C.M., Nutt, L.K., Guo, Y., Jardim, M.J., Thomenius, M.J., Freel, C.D., Darbandi, R., Ahn, J.H., Arroyo, J.D., Wang, X.F.,

Shenolikar, S., Nairn, A.C., Dunphy, W.G., Hahn, W.C., Virshup, D.M., Kornbluth, S., Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell*, 2006. **127**: p. 759-773.

114. Vigneron, S., Brioudes, E., Burgess, A., Labbe, J.C., Lorca, T., Castro, A., Greatwall maintains mitosis through the regulation of PP2A. *EMBO J*, 2009. **28**: p. 2786-2793.

115. Kops, G.J., The kinetochore and spindle checkpoint in mammals. *Front Biosci*, 2008. **13**: p. 3606-3620.

116. Kitajima, T.S., T. Sakuno, K. Ishiguro, S. Iemura, T. Natsume, S.A. Kawashima, et al., Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature*, 2006. **441**(7089): p. 46-52.

117. Gil-Bernabe, A.M., F. Romero, M.C. Limon-Mortes and M. Tortolero, Protein phosphatase 2A stabilizes human securin, whose phosphorylated forms are degraded via the SCF ubiquitin ligase. *Mol Cell Biol*, 2006. **26**(11): p. 4017-27.

118. Ferrigno, P., T.A. Langan and P. Cohen, Protein phosphatase 2A1 is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases. *Mol Biol Cell*, 1993. **4**(7): p. 669-77.

119. Schmitz, M.H., Held, M., Janssens, V., Hutchins, J.R., Hudecz, O., Ivanova, E., Goris, J., Trinkle-Mulcahy, L., Lamond, A.I., Poser, I., Hyman, A.A., Mechtler, K., Peters, J.M., Gerlich, D.W., Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells. *Nat Cell Biol*, 2010. **12**(9): p. 886-893.

120. Mochida, S., Ikeo, S., Gannon, J., Hunt, T., Regulated activity of PP2A-B55delta is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts. *EMBO J*, 2009. **28**: p. 2777-2785.

121. Garcia, A., X. Cayla, J. Guergnon, F. Dessauge, V. Hospital, M.P. Rebollo, et al., Serine/threonine protein phosphatases PP1 and PP2A are key players in apoptosis. *Biochimie*, 2003. **85**(8): p. 721-6.

122. Van Hoof, C., Goris, J., Phosphatases in apoptosis: to be or not to be, PP2A is in the heart of the question. *Biochim Biophys Acta*, 2003. **1640**: p. 97-104.

123. Klumpp, S. and J. Krieglstein, Serine/threonine protein phosphatases in apoptosis. *Current Opinion in Pharmacology*, 2002. **2**(4): p. 458-62.

124. Gausdal, G., Krakstad, C., Herfindal, L., Doskeland, S.O., *Serine/threonine protein phosphatases in apoptosis*, in *Apoptosis, Cell Signaling, and Human Diseases*, R. Srivastava, Editor. 2007, Humana: New York. p. 151-166.

125. Harada, H., Quearry, B., Ruiz-Vela, A., Korsmeyer, S.J., Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *PNAS*, 2004. **101**(43): p. 15313–15317.

126. Green, D.R. and J.C. Reed, Mitochondria and apoptosis. [Review] [59 refs]. *Science*, 1998. **281**(5381): p. 1309-12.

127. Adams, J.M., Cory, S., Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem Sci*, 2001. **26**(1): p. 61-66.

128. Gross, A., McDonnell, J.M., S.J. Korsmeyer, BCL-2 family members and the mitochondria in apoptosis. *Gene Dev*, 1999. **13**: p. 1899-1911.

129. Basu, A., Haldar, S., The relationship between Bcl-2, Bax and p53: consequences for cell sysle progression and cell death. *Mol Hum Reprod*, 1998. **4**(12): p. 1099-1109.

130. Xin, M., Deng, X., Protein phosphatase 2A enhances the proapoptotic function of Bax through dephosphorylation. *J Biol Chem*, 2006. **281**(27): p. 11859-18867.

131. Deng, X., T. Ito, B. Carr, M. Mumby and W.S. May, Jr., Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. *J Biol Chem*, 1998. **273**(51): p. 34157-63.

132. Galadari, S., K. Kishikawa, C. Kamibayashi, M.C. Mumby and Y.A. Hannun, Purification and characterization of ceramide-activated protein phosphatases. *Biochemistry*, 1998. **37**(32): p. 11232-8.

133. Ruvolo, P.P., X. Deng, T. Ito, B.K. Carr and W.S. May, Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. *J Biol Chem*, 1999. **274**(29): p. 20296-300.

134. Ruvolo, P.P., W. Clark, M. Mumby, F. Gao and W.S. May, A functional role for the B56 alpha subunit of protein phosphatase 2A in ceramide-mediated regulation of Bcl2 phosphorylation status and function. *J Biol Chem*, 2002. **2**: p. 2.

135. Chiang, C.W., C. Kanies, K.W. Kim, W.B. Fang, C. Parkhurst, M. Xie, et al., Protein phosphatase 2A dephosphorylation of phosphoserine 112 plays the gatekeeper role for BAD-mediated apoptosis. *Mol Cell Biol*, 2003. **23**(18): p. 6350-62.

136. Dougherty, M.K. and D.K. Morrison, Unlocking the code of 14-3-3. *J Cell Sci*, 2004. **117**(Pt 10): p. 1875-84.

137. Santoro, M.F., R.R. Annand, M.M. Robertson, Y.W. Peng, M.J. Brady, J.A. Mankovich, et al., Regulation of protein phosphatase 2A activity by caspase-3 during apoptosis. *J Biol Chem*, 1998. **273**(21): p. 13119-28.

138. Alvarado-Kristensson, M., Andersson, M., Protein phosphatase 2A regulates apoptosis in neutrophils by dephosphorylating both p38 MAPK and its substrate caspase 3. *J Biol Chem*, 2005. **280**: p. 6238-6244.

139. Yan, L., Lavin, V.A., Moser, L.R., Cui, Q., Kanies, C., Yang, E., PP2A regulates the pro-apoptotic activity of FOXO1. *J Biol Chem*, 2008. **283**(12): p. 7411-7420.

140. Singh, A., Ye, M., Bucur, O., Zhu, S., Santos, M.T., Rabinovitz, I., Wei, W., Gao, D., Hahn, W.C., Khosravi-Far, R., Protein phosphatase 2A reactivates FOXO3a through a dynamic interplay with 14-3-3 and Akt. *Mol Biol Cell*, 2010. **21**: p. 1140-1152.

141. Shouse, G.P., Cai, X., Liu, X., Serine 15 phosphorylation of p53 directs its interaction with B56γ and the tumor suppressor activity of B56γ-specific protein phosphatase 2A. *Mol Cell Biol*, 2008. **28**: p. 448-456.

142. Li, H.H., Cai, X., Shouse, G.P., Piluso, L.G., Liu, X., A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J*, 2007. **26**: p. 402-411.

143. Mi, J., Bolesta, E., Brautigan, D.L., Larner, J.M., PP2A regulates ionizing radiation-induced apoptosis through Ser46 phosphorylation of p53. *Mol Cancer Ther*, 2009. **8**(1): p. 135-140.

144. Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., Reed, J.C., Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene*, 1994. **26**(2): p. 402-411.

145. Fridman, J.S., Lowe, S.W., Control of apoptosis by p53. *Oncogene*, 2003. **22**: p. 9030-9040.

146. Moroni, M.C., Hickman, E.S., Lazzerini-Denchi, E., Caprara, G., Colli, E., Cecconi, F., Müller, H., Helin, K., Apaf-1 is a transcriptional target for E2F and p53. *Nat Cell Biol*, 2001. **3**(6): p. 552-558.

147. Jin, Z., Wallace, L., Harper, S.Q., Yang, J., PP2A:B56epsilon, a substrate of caspase-3, regulates p53-dependent and p53 independent apoptosis during development. *J Biol Chem*, 2010. **285**(45): p. 34493-34502.

148. Deng, X., Gao, F., May, W.S., Protein phosphatase 2A inactivates Bcl2's antiapoptotic function by dephosphorylation and up-regulation of Bcl2-p53 binding. *Blood*, 2009. **113**: p. 422-428.

149. Ades, T., Alteri, R., Bandi, P., Blecher, E., Brooks, D., Casares, C., Center, M., Chen, A., Cokkinides, V., DeSantis, C., Elk, R., Gansler, T., Gapstur, S., Glynn,T.,Graves, K., Jacobs, E., Kramer, J., Lichtenfeld, L., Linet, M., McCullough, M., McNeal, B. et al, *American Cancer Society. Cancer Facts & Figures 2011*. 2011, American Cancer Society: Atlanta, GA.

150. Hanahan, D.a.W., R.A., Hallmarks of cancer: the next generation. *Cell*, 2011. **144**: p. 646-674.

151. Negrini, S., Gorgoulis, V.G., Halazonetis, T.D., Genomic instability-an evolving hallmark of cancer. *Nat Rev Mol Cell Bio*, 2010. **11**: p. 220-228.

152. Colotta, F., Allavena, P., Sica, A., Garlanda, C., Mantovani, A., Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*, 2009. **30**(7): p. 1073-1081.

153. Bialojan, C. and A. Takai, Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem J*, 1988. **256**(1): p. 283-90.

154. Suganuma, M., H. Fujiki, H. Suguri, S. Yoshizawa, M. Hirota, M. Nakayasu, et al., Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc Natl Acad Sci U S A*, 1988. **85**(6): p. 1768-71.

155. Suganuma, M., M. Suttajit, H. Suguri, M. Ojika, K. Yamada and H. Fujiki, Specific binding of okadaic acid, a new tumor promoter in mouse skin. *Febs Lett*, 1989. **250**(2): p. 615-8.

156. Katoh, F., Fitzgerald, P.J., Giroldi, L., Fujiki, H., Sugimura, T., Yamasaki, H., Okadaic acid and phorbol esters: comparative effects of these tumor promotors on cell transformation, intercellular communication snf differentiation in vitro. *Jpn J Cancer Res*, 1990. **81**: p. 590-597.

157. Kim, S.J., R. Lafyatis, K.Y. Kim, P. Angel, H. Fujiki, M. Karin, et al., Regulation of collagenase gene expression by okadaic acid, an inhibitor of protein phosphatases. *Cell Regul*, 1990. **1**(3): p. 269-78.

158. Guenin, S., Schwartz, L., Morvani, D., Steyaert, J.M., Poignet, A., Madelmont, J.C., Demidem, A., PP2A activity is controlled by methylation and regulates oncoprotein expression in melanoma cells: A mechanism which participates in

growth inhibition induced by chloroethylnitrosourea treatment. *Int J Onco*, 2008. **32**: p. 49-57.

159. Andjelkovic, M., T. Jakubowicz, P. Cron, X.F. Ming, J.W. Han and B.A. Hemmings, Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. *Proc Natl Acad Sci U S A*, 1996. **93**(12): p. 5699-704.

160. Switzer, C.H., Cheng, R.Y.S., Vitek, T.M., Christensen, D.J., Wink, D.A., and Vitek, M.P., Targeting SET/I2PP2A oncoprotein functions as a multi-pathway strategy for cancer therapy. *Oncogene*, 2011. **30**(22): p. 2504–2513.

161. Perrotti, D.N., P., ReSETting PP2A tumour suppressor activity in blast crisis and imatinib-resistant chronic myelogenous leukaemia. *British Journal of Cancer*, 2006. **95**: p. 775-781.

162. Neviani, P., R. Santhanam, R. Trotta, M. Notari, B.W. Blaser, S. Liu, et al., The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell*, 2005. **8**(5): p. 355-68.

163. Christensen, D.J., Chen, Y., Oddo, J., Matta, K.M., Neil, J., Davis, E.D., Volkheimer, A.D., Lanasa, M.C., Friedman, D.R., Goodman, B.K., Gockerman, J.P, Diehl, L.F., de Castro, C.M., Moore, J.O., Vitek, M.P., Weinberg, J.B., SET oncoprotein overexpression in B-cell chronic lymphocytic leukemia and non-Hodgkin's lymphoma: a predictor of aggressive disease and new treatment target. *Blood*, 2011. **118**(15): p. 4150-4158.

164. Junttila, M.R., Puustinen, P., Niemelä, M., Ahola, R., Arnold, H., Böttzauw, T., Ala-aho, R., Nielsen, C., Ivaska, J., Taya, Y., Lu, S.L., Lin, S., Chan, E.K., Wang, X.J., Grènman, R., Kast, J., Kallunki, T., Sears, R., Kähäri, V.M., Westermarck, J., CIP2A inhibits PP2A in human malignancies. *Cell*, 2007. **130**: p. 51-62.

165. Wang, S.W., E.D. Esplin, J.L. Li, L. Huang, A. Gazdar, J. Minna, et al., Alterations of the PPP2R1B Gene in Human Lung and Colon Cancer. *Science*, 1998. **282**: p. 284-287.

166. Zhou, J., H.T. Pham, R. Ruediger and G. Walter, Characterization of the Aalpha and Abeta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. *Biochem J*, 2003. **369**(Pt 2): p. 387-98.

167. Calin, G.A., M.G. di Iasio, E. Caprini, I. Vorechovsky, P.G. Natali, G. Sozzi, et al., 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*, 2000. **19**(9): p. 1191-5.

168. Takagi, Y., M. Futamura, K. Yamaguchi, S. Aoki, T. Takahashi and S. Saji, Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. *Gut*, 2000. **47**(2): p. 268-71.

169. Ruediger, R., H.T. Pham and G. Walter, Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. *Oncogene*, 2001. **20**(1): p. 10-5.

170. Ito, A., Y. Koma, K. Watabe, T. Nagano, Y. Endo, H. Nojima, et al., A truncated isoform of the protein phosphatase 2A B56gamma regulatory subunit may

promote genetic instability and cause tumor progression. *Am J Pathol*, 2003. **162**(1): p. 81-91.

171. Chen, W., R. Possemato, K.T. Campbell, C.A. Plattner, D.C. Pallas and W.C. Hahn, Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell*, 2004. **5**(2): p. 127-36.

172. Arroyo, J.D. and W.C. Hahn, Involvement of PP2A in viral and cellular transformation. *Oncogene*, 2005. **24**(52): p. 7746-55.

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

174. Sablina, A.A. and W.C. Hahn, SV40 small T antigen and PP2A phosphatase in cell transformation. *Cancer Metastasis Rev*, 2008. **27**(2): p. 137-46.

175. Pallas, D.C., W. Weller, S. Jaspers, T.B. Miller, W.S. Lane and T.M. Roberts, The third subunit of protein phosphatase 2A (PP2A), a 55-kilodalton protein which is apparently substituted for by T antigens in complexes with the 36- and 63-kilodalton PP2A subunits, bears little resemblance to T antigens. *J Virol*, 1992. **66**(2): p. 886-93.

176. Ruediger, R., D. Roeckel, J. Fait, A. Bergqvist, G. Magnusson and G. Walter, Identification of binding sites on the regulatory A subunit of protein phosphatase 2A for the catalytic C subunit and for tumor antigens of simian virus 40 and polyomavirus. *Mol Cell Biol*, 1992. **12**(11): p. 4872-82.

177. Cho, U.S., Morrone, S., Sablina, A., Arroyo, J.D., Hahn, W.C., Xu, W. (2007) *Structural basis of PP2A inhibition by small T antigen*. PloS Biol **Volume**, e202

178. Hahn, W.C., S.K. Dessain, M.W. Brooks, J.E. King, B. Elenbaas, D.M. Sabatini, et al., Enumeration of the simian virus 40 early region elements necessary for human cell transformation. *Mol Cell Biol*, 2002. **22**(7): p. 2111-23.

179. Mateer, S.C., S.A. Fedorov and M.C. Mumby, Identification of structural elements involved in the interaction of simian virus 40 small tumor antigen with protein phosphatase 2A. *J Biol Chem*, 1998. **273**(52): p. 35339-46.

180. Porras, A., J. Bennett, A. Howe, K. Tokos, N. Bouck, B. Henglein, et al., A novel simian virus 40 early-region domain mediates transactivation of the cyclin A promoter by small-t antigen and is required for transformation in small-t antigen-dependent assays. *J Virol*, 1996. **70**(10): p. 6902-8.

181. Srinivasan, A., A.J. McClellan, J. Vartikar, I. Marks, P. Cantalupo, Y. Li, et al., The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. *Mol Cell Biol*, 1997. **17**(8): p. 4761-73.

182. Frost, J.A., A.S. Alberts, E. Sontag, K. Guan, M.C. Mumby and J.R. Feramisco, Simian virus 40 small t antigen cooperates with mitogen-activated kinases to stimulate AP-1 activity. *Mol Cell Biol*, 1994. **14**(9): p. 6244-52.

183. Watanabe, G., A. Howe, R.J. Lee, C. Albanese, I.W. Shu, A.N. Karnezis, et al., Induction of cyclin D1 by simian virus 40 small tumor antigen. *Proc Natl Acad Sci U S A*, 1996. **93**(23): p. 12861-6.

184. Moreno, C.S., S. Ramachandran, D.G. Ashby, N. Laycock, C.A. Plattner, W. Chen, et al., Signaling and transcriptional changes critical for transformation of human cells by simian virus 40 small tumor antigen or protein phosphatase 2A B56gamma knockdown. *Cancer Res*, 2004. **64**(19): p. 6978-88.

185. Yuan, H., T. Veldman, K. Rundell and R. Schlegel, Simian Virus 40 Small Tumor Antigen Activates AKT and Telomerase and Induces Anchorage-Independent Growth of Human Epithelial Cells. *J Virol*, 2002. **76**(21): p. 10685-91.

186. Andrabi, S., Hwang, J.H., Choe, J.K., Roberts, T.M., Schaffhausen, B.S., Comparisons between polyoma and SV40 show significant differences in small T antigen function. *J Virol*, 2011. **85**: p. 10649 - 10658.

187. Sontag, J.M. and E. Sontag, Regulation of cell adhesion by PP2A and SV40 small tumor antigen: An important link to cell transformation. *Cell Mol Life Sci*, 2006. **63**: p. 2979-91.

188. Sontag, E., J.M. Sontag and A. Garcia, Protein phosphatase 2A is a critical regulator of protein kinase C zeta signaling targeted by SV40 small t to promote cell growth and NF-kappaB activation. *Embo J*, 1997. **16**(18): p. 5662-71.

189. Nunbhakdi-Craig, V., L. Craig, T. Machleidt and E. Sontag, Simian virus 40 small tumor antigen induces deregulation of the actin cytoskeleton and tight junctions in kidney epithelial cells. *J Virol*, 2003. **77**(5): p. 2807-18.

190. Reece, K.M., Mazalouskasa,M.D., Wadzinski, B.E., The Bα and Bδ regulatory subunits of PP2A are necessary for assembly of the CaMKIV•PP2A signaling complex. *Biochem Biophys Res Comm*, 2009. **386**(4): p. 582-587.

191. Yeh, E., M. Cunningham, H. Arnold, D. Chasse, T. Monteith, G. Ivaldi, et al., A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol*, 2004. **6**(4): p. 308-18.

192. Arnold, H.K., Sears, R.C., Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. *Mol Biol Cell*, 2006. **26**(7): p. 2832-44.

193. Hirakawa, T., Ruley, H.E., Rescue of cells from ras oncogene-induced growth arrest by a second, complementing, oncogene. *PNAS*, 1988(85): p. 1519-1523.

194. Michalovitz, D., Fischer-Fantuzzi, L., Vesco, C., Pipas, J.M., Oren, M., Activated Ha-ras can cooperate with defective simian virus 40 in the transformation of nonestablished rat embryo fibroblasts. *J Virol*, 1987. **61**: p. 2648-2654.

195. Fluck, M.M., Schaffhausen, B.S., Lessons in signaling and tumorigenesis from polyomavirus middle T antigen. *Microbiol Mol Biol Rev*, 2009. **73**(3): p. 542-563.

196. Ruediger, R., K. Fields and G. Walter, Binding specificity of protein phosphatase 2A core enzyme for regulatory B subunits and T antigens. *J Virol*, 1999. **73**(1): p. 839-42.

197. Soeda, E., Arrand, J.R., Smolar, N., Griffin, B.E., Sequence from early region of polyoma virus DNA containing viral replication origin and encoding small, middle and (part of) large T antigens. *Cell*, 1979. **17**: p. 357-370.

198. Carmichael, G.G., B.S. Schaffhausen, D.I. Dorsky, D.B. Oliver and T.L. Benjamin, Carboxy terminus of polyoma middle-sized tumor antigen is required for attachment to membranes, associated protein kinase activities, and cell transformation. *Proc Natl Acad Sci U S A*, 1982. **79**(11): p. 3579-83.

199. Treisman, R., U. Novak, J. Favaloro and R. Kamen, Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. *Nature*, 1981. **292**(5824): p. 595-600.

200. Cheng, J., DeCaprio, J.A., Fluck, M.M., Schaffhausen, B.S., Cellular transformation by simian virus 40 and murine polyoma virus T antigens. *Semin Cancer Biol*, 2009. **19** (4): p. 218-228.

201. Ogris, E., I. Mudrak, E. Mak, D. Gibson and D.C. Pallas, Catalytically inactive protein phosphatase 2A can bind to polyomavirus middle tumor antigen and support complex formation with pp60(c-src). *J Virol*, 1999. **73**(9): p. 7390-8.

202. Brewster, C.E., H.R. Glover and S.M. Dilworth, pp60c-src binding to polyomavirus middle T-antigen (MT) requires residues 185 to 210 of the MT sequence. *J Virol*, 1997. **71**(7): p. 5512-20.

203. Campbell, K.S., K.R. Auger, B.A. Hemmings, T.M. Roberts and D.C. Pallas, Identification of regions in polyomavirus middle T and small t antigens important for association with protein phosphatase 2A. *J Virol*, 1995. **69**(6): p. 3721-8.

204. Talmage, D.A., R. Freund, A.T. Young, J. Dahl, C.J. Dawe and T.L. Benjamin, Phosphorylation of middle T by pp60c-src: a switch for binding of phosphatidylinositol 3-kinase and optimal tumorigenesis. *Cell*, 1989. **59**(1): p. 55-65.

205. Dahl, J., A. Jurczak, L.A. Cheng, D.C. Baker and T.L. Benjamin, Evidence of a role for phosphatidylinositol 3-kinase activation in the blocking of apoptosis by polyomavirus middle T antigen. *J Virol*, 1998. **72**(4): p. 3221-6.

206. Dilworth, S.M., Polyoma virus middle T antigen and its role in identifying cancer-related molecules. *Nat Rev Cancer*, 2002. **2**: p. 1-6.

207. Summers, S.A., L. Lipfert and M.J. Birnbaum, Polyoma middle T antigen activates the Ser/Thr kinase Akt in a PI3-kinase-dependent manner. *Biochem Biophys Res Commun*, 1998. **246**(1): p. 76-81.

208. Meili, R., P. Cron, B.A. Hemmings and K. Ballmer-Hofer, Protein kinase B/Akt is activated by polyomavirus middle-T antigen via a phosphatidylinositol 3-kinase-dependent mechanism. *Oncogene*, 1998. **16**(7): p. 903-7.

209. Rodriguez-Viciana, P., C. Collins and M. Fried, Polyoma and SV40 proteins differentially regulate PP2A to activate distinct cellular signaling pathways involved in growth control. *Proc Natl Acad Sci U S A*, 2006. **103**(51): p. 19290-5.

210. Ogris, E., I. Mudrak and E. Wintersberger, Polyomavirus large and small T antigens cooperate in induction of the S phase in serum-starved 3T3 mouse fibroblasts. *J Virol*, 1992. **66**(1): p. 53-61.

211. Schuchner, S. and E. Wintersberger, Binding of polyomavirus small T antigen to protein phosphatase 2A is required for elimination of p27 and support of S-phase induction in concert with large T antigen [In Process Citation]. *J Virol*, 1999. **73**(11): p. 9266-73.

212. Schuchner, S., M. Nemethova, A. Belisova, B. Klucky, W. Holnthoner and E. Wintersberger, Transactivation of murine cyclin A by polyomavirus large and small T antigens. *J Virol*, 2001. **75**(14): p. 6498-507.

213. Mullane, K.P., M. Ratnofsky, X. Cullere and B. Schaffhausen, Signaling from polyomavirus middle T and small T defines different roles for protein phosphatase 2A. *Mol Cell Biol*, 1998. **18**(12): p. 7556-64.

214. Yen, A., L. Placanica, S. Bloom and S. Varvayanis, Polyomavirus Small t Antigen Prevents Retinoic Acid-Induced Retinoblastoma Protein Hypophosphorylation and Redirects Retinoic Acid-Induced G(0) Arrest and Differentiation to Apoptosis. *J Virol*, 2001. **75**(11): p. 5302-5314.

215. Ventura, J.J., Nebreda, A.R., Protein kinases and phosphatases as therapeutic targets in cancer. *Clin Tranls Oncol*, 2006. **8**(3): p. 153-160.

216. Nunbhakdi-Craig, V., S. Schuechner, J.M. Sontag, L. Montgomery, D.C. Pallas, C. Juno, et al., Expression of protein phosphatase 2A mutants and silencing of the regulatory Balpha subunit induce a selective loss of acetylated and detyrosinated microtubules. *J Neurochem*, 2007. **101**(4): p. 959-71.

217. Kanda, T., K.F. Sullivan and G.M. Wahl, Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr Biol*, 1998. **8**(7): p. 377-85.

218. Kitamura, T., W. Ogawa, H. Sakaue, Y. Hino, S. Kuroda, M. Takata, et al., Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol Cell Bio*, 1998. **18**(7): p. 3708-17.

219. Pallas, D.C., C. Schley, M. Mahoney, E. Harlow, B.S. Schaffhausen and T.M. Roberts, Polyomavirus small t antigen: overproduction in bacteria, purification, and utilization for monoclonal and polyclonal antibody production. *J Virol*, 1986. **60**(3): p. 1075-84.

220. Jackson, J.B., Pallas, D.C., Circumventing cellular control of PP2A by methylation promotes transformation in an Akt-dependent manner. *Neoplasia*, 2012. **14**(7): p. 585-599.

221. Freedman, V.H. and S. Shin, Cellular tumorigenicity in nude mice: Correlation with cell growth in semi-solid medium. *Cell*, 1974. **3**(4): p. 355-59.

222. Vachon, P.H., Integrin signaling, cell survival, and anoikis: Distinctions, differences, and differentiation. *J Signal Transduction*, 2011. **2011**: p. Article ID 738137.

223. Peterson, R.T. and S.L. Schreiber, Translation control: Connecting mitogens and the ribosome. *Curr Biol*, 1998. **8**(7): p. R248-50.

224. Lantier, L., R. Mounier, J. Leclerc, M. Pende, M. Foretz and B. Viollet, Coordinated maintenance of muscle cell size control by AMP-activated protein kinase. *FASEB J*, 2010. **24**: p. 3555-61.

225. Mamane, Y., E. Petroulakis, O. LeBacquer and N. Sonenberg, mTOR, translation initiation and cancer. *Oncogene*, 2006. **25**: p. 6416-22.

226. Li, S.P., C. Brignole, R. Marcellus, S. Thirlwell, O. Binda, M.J. McQuoid, et al., The adenovirus E4orf4 protein induces G2/M arrest and cell death by blocking protein phosphatase 2A activity regulated by the B55 subunit. *J Virol*, 2009. **83**(17): p. 8340-52.

227. Nho, R.S. and M. Peterson, Eukaryotic translation initiation factor 4E binding protein 1 (4EBP-1) function is suppressed by src and protein phosphatase 2A (PP2A) on extracellular matrix. *J Biol Chem*, 2011. **286**(37): p. 31953-65.

228. Nho, R.S. and J. Kahm, Beta1-integrin-collagen interaction suppresses FoxO3a by the coordination of Akt and PP2A. *J Biol Chem*, 2010. **285**(19): p. 14195-209.

229. Kiely, P.A., G.S. Baillie, M.J. Lynch, M.D. Houslay and R. O'Conner, Tyrosine 302 in RACK1 is essential for insulin-like growth factor-I-mediated competitive binding of PP2A and B1 integrin and for tumor cell proliferation and migration. *J Biol Chem*, 2008. **283**(34): p. 22952-61.

230. Xia, H., R. Nho, J. Kleidon, J. Kahm and C.A. Henke, Polymerized collagen inhibits fibroblast proliferation via a mechanism involving the formation of a B1 integrin-protein phosphatase 2A-tuberous sclerosis complex 2 complex that suppresses S6K1 activity. *J Biol Chem*, 2008. **283**(29): p. 20350-60.

231. De Toni-Costes, F., M. Despeaux, J. Bertrand, E. Bourogaa, L. Ysebaert, B. Payrastre, et al., A new a5b1 integrin-dependent survival pathway through GSK3B activation in leukemic cells. *PLoS ONE*, 2010. **5**(3): p. e9807.

232. Gendron, S., J. Couture and F. Aoudjit, Integrin {alpha}2{beta}1 Inhibits Fas-mediated Apoptosis in T Lymphocytes by Protein Phosphatase 2A-dependent Activation of the MAPK/ERK Pathway. *J Biol Chem*, 2003. **278**(49): p. 48633-48643.

233. Chen, J., B.L. Martin and D.L. Brautigan, Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science*, 1992. **257**(5074): p. 1261-4.

234. Ramel, D., F. Lagarrigue, S. Dupruis-Coronas, G. Chicanne, N. Leslie, F. Gaits-Iacovoni, et al., PtdIns5P protects Akt from dephosphorylation through PP2A inhibition. *Biochem Biophys Res Commun* 2009. **387**(1): p. 127-31.

235. Bielinski, V.A. and M.C. Mumby, Functional analysis of the PP2A subfamily of protein phosphatases in regulating drosophila S6 kinase. *Exp Cell Res*, 2007. **313**(14): p. 3117-26.

236. Hombauer, H., D. Weismann, I. Mudrak, C. Stanzel, T. Fellner, D.H. Lackner, et al., Generation of active protein phosphatase 2A is coupled to holoenzyme assembly. *PLoS Biol*, 2007. **5**(6): p. e155.

237. Ortega-Gutierrez, S., D. Leung, S. Ficarro, E.C. Peters and B.F. Cravatt, Targeted disruption of the pme-1 gene causes loss of demethylated PP2A and perinatal lethality in mice. *PLoS ONE*, 2008. **3**(7): p. e2486.

238. Puustinen, P., M.R. Junttila, S. Vanhatupa, A.A. Sablina, M.E. Hector, K. Teittinen, et al., Pme-1 protects extracellular signal-regulated kinase pathway activity from protein phosphatase 2A-mediated inactivation in human malignant glioma. *Cancer Res*, 2009. **69**(7): p. 2870-7.

239. Gillet, R., C. Cavard, G. Grimber, P. Briand and V. Joulin, Hepatic expression of SV40 small-T antigen blocks the in vivo CD95-mediated apoptosis. *Biochem Biophys Res Comm*, 2001. **284**: p. 369-76.

240. Zhao, J.J., O.V. Gjoerup, R.R. Subramanian, Y. Cheng, W. Chen, T.M. Roberts, et al., Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. *Cancer Cell*, 2003. **3**(5): p. 483-95.

241. Kennedy, I.M. and D.P. Leader, Increased phosphorylation of ribosomal protein S6 in hamster fibroblasts transformed by polyoma virus and simian virus 40. *Biochem J*, 1981. **198**: p. 235-37.

242. Blenis, J. and R.L. Erikson, Phosphorylation of the ribosomal protein S6 is elevated in cells transformed by a variety of tumor viruses. *J Virol*, 1984. **50**(3): p. 966-69.

243. Talmage, D.A., J. Blenis and T.L. Benjamin, Polyomavirus middle T antigen induces ribosomal protein S6 phosphorylation through pp60c-src-dependent and -independent pathways. *Mol Cell Biol*, 1988. **8**(6): p. 2309-15.

244. Kelley, W.L. and S.J. Landry, Chaperone power in a virus? *TIBS*, 1994. **19**: p. 277-278.

245. Tsai, J., Douglas, M.G., A conserved HPD sequence of the J-domain is necessary for YDI1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. *J Biol Chem*, 1996. **271**: p. 9347-9354.

246. Berjanskii, M.V., M.I. Riley, A. Xie, V. Semenchenko, W.R. Folk and S.R. Van Doren, NMR Structure of the N-terminal J Domain of Murine Polyomavirus T Antigens. IMPLICATIONS FOR DnaJ-LIKE DOMAINS AND FOR MUTATIONS OF T ANTIGENS. *J Biol Chem*, 2000. **275**(46): p. 36094-36103.

247. Kim, H.Y., Ahn, B.Y., Cho, Y., Structural basis for the inactivation of retinoblastoma tumor suppressor by SV40 large T antigen. *EMBO J*, 2001. **20**: p. 295-304.

248. Hartl, F.U., Molecular chaperones in molecular protein folding. *Nature*, 1996. **381**: p. 571-580.

249. Kampinga, H.H., Craig, E.A., The Hsp70 chaperone machinery: J proteins as drivers of functional specificity. *Mol Cell Bio*, 2010. **11**: p. 579-592.

250. Mayer, M.P., Bukau, B., Hsp70 chaperones: Cellular functions and molecular mechanisms. *Cell Mol Life Sci*, 2005. **62**: p. 670-684.

251. Jäättelä, M., Heat shock proteins as cellular lifeguards. *Ann Med*, 1999. **31**(4): p. 261-271.

252. Vargas-Roig, L.M., Fanelli, M.A., López, L.A., Gago, F.E., Tello, O., Aznar, J.C., Ciocca, D.R., Heat shock proteins and cell proliferation in human breast cancer biopsy samples. *Cancer Detect Prev*, 1997. **21**(5): p. 441-451.

253. Ciocca, D.R., Clark, G.M., Tandon, A.K., Fuqua, S.A., Welch, W.J., McGuire, W.L., Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: prognostic implications. *J Natl Cancer Inst*, 1993. **85**(7): p. 570-574.

254. Jolly, C., Morimoto, R.I., Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J Natl Cancer Inst*, 2000. **92**: p. 1564–1572.

255. Pocaly, M., Lagarde, V., Etienne, G., Ribeil, J.A., Claverol, S., Bonneu, M., Moreau-Gaudry, F., Guyonnet-Duperat, V., Hermine, O., Melo, J.V., Dupouy, M., Turcq, B., Mahon, F.X., Pasquet, J.M., Overexpression of the heat-shock protein 70 is associated to imatinib resistance in chronic myeloid leukemia. *Leukemia*, 2007. **21**: p. 93-101.

256. Taldone, T., Gozman, A., Maharaj, R., Chiosis, G., Targeting Hsp90: small-molecule inhibitors and their clinical development. *Curr Opin Pharmacol*, 2008. **8**: p. 370-374.

257. Leu, J.I., Pimkina, J., Frank, A., Murphy, M.E., George, D.L., A small molecule inhibitor of inducible heat shock protein 70. *Mol Cell*, 2009. **36**: p. 15-27.

258. MacKeigan, J.P., Murphy, L.O., Blenis, J., Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nat Cell Biol*, 2005. **7**(6): p. 591-600.

259. Castilho, P.V., Williams, B.C., Mochida, S., Zhao, Y, Goldberg, M.L., The M Phase Kinase Greatwall (Gwl) Promotes Inactivation of PP2A/B55delta, a Phosphatase Directed Against CDK Phosphosites. *Mol Biol Cell*, 2009. **20**: p. 4777-4789.

260. Medema, R.H., Macůrek, L., Checkpoint recovery in cells: how a molecular understanding can help in the fight against cancer. *F1000 Biology Reports*, 2011. **3**: p. article 10.

261. Peng, A., Yamamoto,T.M., Goldberg, M.L. and Maller, J.L., A novel role for Greatwall kinase in recovery from DNA damage. *Cell Cycle*, 2010. **9**(21): p. 4364-4269.

262. Gaillard, S., K.M. Fahrbach, R. Parkati and K. Rundell, Overexpression of simian virus 40 small-T antigen blocks centrosome function and mitotic progression in human fibroblasts. *J Virol*, 2001. **75**(20): p. 9799-807.

263. Gjoerup, O., D. Zaveri and T.M. Roberts, Induction of p53-independent apoptosis by simian virus 40 small t antigen. *J Virol*, 2001. **75**(19): p. 9142-55.

264. Scheffner, M., K. Munger, J.C. Byrne and P.M. Howley, The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci U S A*, 1991. **88**(13): p. 5523-7.

265. Mumby, M., A new role for protein methylation: switching partners at the phosphatase ball. *Sci STKE*, 2001. **2001**(79): p. PE1.

266. Wepf, A., Glatter, T., Schmidt, A., Aebersold, R., Gstaiger, M., Quantitative interaction proteomics using mass spectrometry. *Nat Methods*, 2009. **6**(3): p. 203-205.

267. Calvo, E., V. Bolos and E. Grande, Multiple roles and therapeutic implications of Akt signaling and cancer. *OncoTargets and Therapy*, 2009. **2**: p. 135-50.

268. Jastrzebski, K., K.M. Hannan, E.B. Tchhoubrieva, R.D. Hannan and R.B. Pearson, Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. *Growth Factors*, 2007. **25**(4): p. 209-26.

269. Nozawa, H., T. Watanabe and H. Nagawa, Phosphorylation of ribosomal p70 S6 kinase and rapamycin sensitivity in human colorectal cancer. *Cancer Letters*, 2007. **251**: p. 105-13.

270. Zhou, Y., Y. Pan, S. Zhang, X. Shi, T. Ning and Y. Ke, Increased phosphorylation of p70 S6 kinase is associated with HPV16 infection in cervical cancer and esophageal cancer. *British Journal of Cancer*, 2007. **97**: p. 218-22.

271. Castellvi, J., A. Garcia, F. Rojo, C. Ruiz-Marcellan, A. Gil, J. Baselga, et al., Phosphorylated 4E binding protein 1: A hallmark of cell signaling that correlates with survival in ovarian cancer. *Cancer*, 2006. **107**(8): p. 1801-11.

272. Kwon, H.K., G.U. Bae, J.W. Yoon, Y.K. Kim, H.Y. Lee, H.W. Lee, et al., Constitutive activation of p70 S6K in cancer cells. *Arch Pharm Res*, 2002. **25**(5): p. 685-90.

273. Barlund, M., F. Forozan, J. Kononen, L. Bubendorf, Y. Chen, M.L. Bittner, et al., Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. *J NCI*, 2000. **92**(15): p. 1252-59.

274. Bachovchin, D.A., Mohr, J.T., Speers, A.E., Wang, C., Berlin, J.M., Spicer, T.P., Fernandez-Vega, V., Chase, P., Hodder, P.S., Schürer, S.C., Nomura, D.K., Rosen, H., Fu, G.C., Cravatt, B.F., Academic cross-fertilization by public screening yields a remarkable class of protein phosphatase methylesterase-1 inhibitors. *PNAS*, 2012. **108**(17): p. 6811-6816.