Turun yliopisto
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THE ROLE OF PME-1 IN CANCER: THERAPEUTIC IMPLICATIONS

Amanpreet Kaur

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1238 | Medica - Odontologica | Turku 2016

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Cover photo: Amanpreet Kaur

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6532-8 (PRINT)
ISBN 978-951-29-6533-5 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy - Turku, Finland 2016

To my family...

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The Role of PME-1 in Cancer: Therapeutic Implications

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ABSTRACT

Protein phosphatase 2A (PP2A) plays a major role in maintaining cellular signaling homeostasis in human cells by reversibly affecting the phosphorylation of a variety of proteins. Protein phosphatase methylesterase-1 (PME-1) negatively regulates PP2A activity by reversible demethylation and active site binding. Thus far, it is known that overexpression of PME-1 in human gliomas contributes to ERK pathway signaling, cell proliferation, and malignant progression. Whether PME-1-mediated PP2A inhibition promotes therapy resistance in gliomas is unknown. Specific PP2A targets regulated by PME-1 in cancers also remain elusive. Additionally, whether oncogenic function of PME-1 can be generalized to various human cancers needs to be investigated.

This study demonstrated that PME-1 expression promotes kinase inhibitor resistance in glioblastoma (GBM). PME-1 silencing sensitized GBM cells to a group of clinically used indolocarbazole multikinase inhibitors (MKIs). To facilitate the quantitative evaluation of MKIs by cancer-cell specific colony formation assay, Image-J software-plugin 'ColonyArea' was developed. PME-1-silencing was found to reactivate specific PP2A complexes and affect PP2A-target histone deacetylase HDAC4 activity. The HDAC4 inhibition induced synthetic lethality with MKIs similar to PME-1 depletion. However, synthetic lethality by both approaches required co-expression of a pro-apoptotic protein BAD. In gliomas, PME-1 and HDAC4 expression was associated with malignant progression. Using tumor PME-1, HDAC4 and BAD expression based stratification signatures this study defined patient subgroups that are likely to respond to MKI alone or in combination with HDAC4 inhibitor therapies.

In contrast to the oncogenic role of PME-1 in certain cancer types, this study established that colorectal cancer (CRC) patients with high tumor PME-1 expression display favorable prognosis. Interestingly, PME-1 regulated survival signaling did not operate in CRC cells. Summarily, this study potentiates the candidacy of PME-1 as a therapy target in gliomas, but argues against generalization of these findings to other cancers, especially CRC.

KEYWORDS: PME-1, PP2A, HDAC4, glioma, kinase inhibitor, ColonyArea, colorectal cancer biomarker, TCGA

Amanpreet Kaur

PME-1:n vaikutukset syövässä ja niiden terapeuttinen potentiaali

Turun Yliopisto, Lääketieteellinen tiedekunta, Patologian laitos, Turun Molekyylilääketieteen tohtoriohjelma ja Biolääketieteen tohtoriohjelma, Turun Biotekniikan Keskus, Turku, Suomi

TIIVISTELMÄ

Proteiinifosfataasi 2A (PP2A)-entsyymillä on tärkeä rooli solun sisäisen signaloinnin säätelyssä sillä se defosforyloi lukuisia signalointi-proteiineja. Proteiinifosfataasi metyyliesteraasi-1 (PME-1)-entsyymi taas säätelee negatiivisesti PP2A:n aktiivisuutta demetyloimalla sitä. Ihmisen glioomassa PME-1:n korkea ilmentyminen lisää syöpää edistävän ERK-signalointireitin aktiivisuutta syöpäsoluissa, syöpäsolujen jakautumista ja edelleen syövän pahanlaatuisuutta. Toistaiseksi on vielä selvittämättä, edistääkö PME-1:n välittämä PP2A:n esto terapiaresistenssiä glioomassa. Lisäksi PME-1:n säätelemät spesifiset PP2A:n kohde-proteiinit syövässä ovat vielä tuntemattomia. Lisätutkimuksia myös kaivataan PME-1:n roolista eri syöpätyypeissä.

Tämä tutkimus osoitti, että PME-1:n ilmentyminen lisää resistenssiä kinaasi-inhibiittoreille glioblastoomassa (GBM). PME-1-geenin hiljentäminen altisti GBM-solut indolokarbatsoli ryhmän multikinaasi-inhibiittoreille (MKI) jotka ovat kliinisessä kehityksessä muihin syöpä-tyyppeihin. Parantaaksemme MKI-yhdisteiden vaikutusten arviointia pesäkemuodostukseen syöpäsoluviljelmissä, kehitimme “ColonyArea” lisäosan Image-J-ohjelmaan. Osoitimme, että PME-1:n hiljentäminen lisää spesifisten PP2A-kompleksien aktiivisuutta ja säätelee PP2A:n kohdeproteiinia, histonideasetylaasia (HDAC4). Kuten PME-1:n hiljen-täminen, myös HDAC4:n esto yhdessä MKI-yhdisteiden kanssa indusoi synteettistä letaalisuutta glioomasoluissa. Kumpikin mekanismi vaatii apoptoosia edistävän BAD-proteiinin ilmentymisen. PME-1:n ja HDAC4:n ilmentyminen korreloi glioman etenemiseen. Kasvaimien PME-1, HDAC4 ja BAD ilmentymistasojen perusteella voitaisiinkin mahdollisesti erotella glioomapotilaat, jotka reagoivat MKI-terapiaan joko HDAC4-inhibiittoriterapian kanssa tai ilman.

Päinvastoin kuin tietyissä muissa syöpätyypeissä, korkea PME-1:n ilmentyminen paransi potilaiden ennustetta paksusuolen syövässä. PME-1 ei lisännyt selviytymistä edistävien signalointireittien aktiivi-suutta paksusuolen syöpäsoluissa. Yhteenvetona tämä tutkimus osoittaa PME-1:n olevan potentiaalinen lääkehoidon kohde glioman hoidossa, mutta tämä löydös ei ole välttämättä yleistettävissä muiden syöpien, tai ei ainakaan paksusuolen syövän hoidossa.

AVAINSANAT: PME-1, PP2A, HDAC4, glioma, kinaasi-inhibiittori, ColonyArea, paksusuolen syövän biomarkkeri, TCGA

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ABBREVIATIONS

ALL	Acute Lymphoblastic Leukemia
AML	Acute myeloid leukemia
BAD	Bcl-2-associated death promoter
BBB	Blood-brain barrier
BCL	B-cell lymphoma
CaMK	Calcium calmodulin-dependent kinase
CDK	Cyclin-dependent kinase
CHK	Checkpoint Kinase
CIP2A	Cancerous inhibitor of protein phosphatase 2A
CNA	Copy number alteration
CNS	Central nervous system
CRC	Colorectal cancer
DFS	Disease-free survival
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EGFRi	EGFR inhibitor
ERK	Extracellular signal-regulated kinase
FUS	Focused ultrasound
GBM	Glioblastoma (Glioblastoma multiforme)
GSC	Glioma or glioblastoma stem cell
GSK-3β	Glycogen synthase kinase 3 beta
HCC	Hepatocellular carcinoma
HEK	Human embryonic kidney cells
HDAC4	Histone deacetylase 4
HDACi	HDAC inhibitor
HIF1α	Hypoxia inducible factor 1α
H-Ras or *HRAS*	Harvey rat sarcoma viral oncogene homolog
IHC	Immunohistochemistry
KO	Knockout
K-Ras or *KRAS*	Kirsten rat sarcoma viral oncogene homolog
Leu	Leucine
LCMT-1	Leucine carboxyl methyltransferase-1
LGG	Low-grade glioma
MAPK	Mitogen-activated protein kinases
MBs	Microbubbles
MDM	Mouse double minute 2 homolog
MEF2	Myocyte enhancer factor-2
MEFs	Mouse embryonic fibroblast cells
MEK	Mitogen-activated protein kinase kinase
MKI	Multikinase inhibitor
Mn^{2+}	Manganese ions
mTOR	mammalian target of rapamycin

NLS	Nuclear localization signal
OA	Okadaic acid
OS	Overall survival
PDGFR	Platelet-derived growth factor receptor
PI3K or *PIK3A*	Phosphatidylinositol 3-kinase
PK(A, G, C)	Protein kinase (A, G, C)
PME-1 or *PPME1*	Protein phosphatase methylesterase 1
PP2A	Protein phosphatase 2A
PP2A-A	Protein phosphatase 2A A-subunit
PP2A-C	Protein phosphatase 2A C-subunit
PSTP	Protein Serine/Threonine phosphatase
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
PTPA	Protein phosphatase 2A activator
PTyP	Protein Tyrosine phosphatase
R2	PPP2R2 family PP2A B-subunits
R5	PPP2R5 family PP2A B-subunits
R3	PPP2R3 family PP2A B-subunits
RB or pRb	Retinoblastoma protein
RTK	Receptor tyrosine kinase
Runx	Runt-related transcription factor
S6K or RPS6K	Ribosomal protein S6 Kinase
Ser	Serine
SET	Inhibitor-2 of protein phosphatase 2A
SIK	Salt-inducible kinase
siRNA	silencing RNA
STS	Staurosporine
TCGA	The Cancer Genome Atlas
Thr	Threonine
TIPRL1	Target of rapamycin signaling pathway regulator-like 1
TKi	Tyrosine kinase inhibitor
TMZ	Temozolomide
TP53 or p53	Tumor protein 53
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
WHO	World health organization

LIST OF ORIGINAL PUBLICATIONS

I. Guzmán C*, Bagga M*, **Kaur A**, Westermarck J, Abankwa D. ColonyArea: An ImageJ plugin to automatically quantify colony formation in clonogenic assays. *PLoS One,* 2014; 9(3): e92444

II. **Kaur A**, Denisova O.V, Jumppanen M, Peuhu E, Qiao X, Ahmed S.U, Raheem O, Haapasalo H, Eriksson J, Chalmers A.J, Laakkonen P, Westermarck J. PP2A inhibitor PME-1 drives kinase inhibitor resistance in glioblastoma. (*Manuscript submitted*, 2016)

III. **Kaur A***, Elzagheid A*, Birkman EM, Avoranta T, Kytölä V, Korkeila E, Syrjänen K, Westermarck J, Sundström J. Protein phosphatase methylesterase-1 (PME-1) expression predicts a favourable clinical outcome in colorectal cancer. *Cancer Medicine*, 2015; 4(12): 1798–1808

* denotes equal contribution

1. INTRODUCTION

Protein phosphorylation plays an important role in the cellular signaling, by conveying cell proliferation, survival, and death signals across the cellular machinery. Protein kinases and phosphatases regulate the phosphorylation status of thousands of proteins in a cell, and thereby maintain a state of homeostasis necessary for normal cell function (Brautigan, 2013; Shi, 2009). However, in cancerous cells this homeostasis is disrupted by various molecular changes leading to an increased flux through the pro-survival signaling pathways, which promote malignant growth (Hanahan & Weinberg, 2011). A limited number of molecular alterations contribute to generation of most cancers (Hahn & Weinberg, 2002). Among these alterations, inactivation of protein phosphatase 2A (PP2A) serves as a prerequisite for the malignant transformation of human cells (Hahn et al, 2002). PP2A inhibition in cancers occurs by genetic alterations in its subunit genes in a small fraction of cases, and by overexpression of PP2A inhibitory proteins, such as CIP2A (cancerous inhibitor of PP2A), SET (inhibitor-2 of PP2A) and PME-1 (protein phosphatase methylesterase 1) in the remaining majority of cases (Haesen et al, 2012; Westermarck & Hahn, 2008). PP2A is a trimeric enzyme that comprises of a catalytic (C), a scaffolding (A), and a regulatory (B) subunit. More than twenty different B-subunits are known which restrict the PP2A phosphatase activity to a limited number of target proteins (Eichhorn et al, 2009; Sangodkar et al, 2015). However, distinct PP2A (B-subunit) complexes altogether can dephosphorylate a plethora of phosphoproteins.

Protein phosphatase methylesterase-1 (PME-1) is an enzymatic protein, which reversibly removes a methyl group modification from the PP2A-C subunit (Ogris et al, 1999). PME-1 can also bind PP2A-C in the active site, release catalytic metal ions, and inhibit PP2A activity (Xing et al, 2008). Based on these functions, PME-1 regulates PP2A activity towards different target proteins by altering the binding of certain B-subunits to the PP2A core (AC dimer) complex (Janssens et al, 2008; Sents et al, 2013). Overexpression of PME-1 occurs in nearly half of human astrocytic gliomas, endometrial cancers and in a small fraction of gastric and lung cancers (Li et al, 2014; Puustinen et al, 2009; Wandzioch et al, 2014). In these tumors, PME-1 promotes cell survival by inhibiting the PP2A activity towards mitogen activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) and/or phosphoinositide 3-kinase (PI3K)/AKT signaling pathways. High PME-1 expression also correlates with the malignant progression of astrocytic gliomas (Puustinen et al, 2009). The expression and function of PME-1 in other

cancers needs to be investigated. The identity of specific PP2A targets regulated by PME-1 is also obscure.

One of the major types of brain tumors is astrocytic glioma, and the most commonly occurring adult glioma is the highly aggressive primary glioblastoma (GBM). Molecular alterations in GBM are well characterized and those leading to activation of receptor tyrosine kinase (RTK)/PI3K/AKT pathway are highly prevalent (Brennan et al, 2013). Paradoxically, kinase inhibitor therapies targeting this pathway have failed to significantly improve GBM patient survival, which remains as low as 15 months (median overall survival) (Cloughesy et al, 2014; Reardon et al, 2014). An understanding of the mechanisms leading to kinase inhibitor resistance in GBM is desperately required in order to design better therapies and/or to identify the subset of patients sensitive to these therapies.

This thesis examined the relevance of PME-1 expression in mediating kinase inhibitor resistance in GBM. This study also identified a protein lysine deacetylase HDAC4 (histone deacetylase 4) as a novel PME-1-regulated PP2A target, and further demonstrated its function in promoting kinase inhibitor resistance in GBM. The expression of these therapy resistance factors (PME-1 and HDAC4), and Bcl-2-associated death promoter (BAD) protein was examined in a panel of clinical astrocytic glioma samples. Based on this, subsets of glioma patients were identified that are likely to benefit from kinase inhibitor therapies. Additionally, the expression of PME-1 and its possible role as a prognostic factor in colorectal cancers (CRCs) was studied. This study demonstrated an unexpected role of PME-1 as a marker of better prognosis in CRC.

2. REVIEW OF THE LITERATURE

2.1. Cancer

Cancer is group of more than a hundred different types of diseases that are characterized by the uncontrolled growth of abnormal cells in the body with a potential to invade nearby or distant tissues (Hanahan & Weinberg, 2000) (www.cancer.gov). According to the world health organization (WHO) estimates in 2011, cancer is the leading cause of deaths worldwide (Torre et al, 2015). Cancer burden is expected to rise during the coming years due to increasing age, adoption of lifestyle behaviors associated with higher cancer risk, and other demographic transitions associated with economic development (Ferlay et al, 2015; Torre et al, 2015). According to GLOBOCAN global cancer statistics in 2012, about 14 million cancer cases were diagnosed, which lead to 8.2 million deaths annually in the world (Ferlay et al, 2015; Torre et al, 2015). It has been estimated that the annual cancer incidence would increase to 20 million new cases in 2025 (Ferlay et al, 2015).

2.1.1. Hallmarks of cancer

Cancers are generated in a multistep process, wherein the cells acquire a number of genetic and epigenetic alterations in proto-oncogenes and tumor-suppressor genes over a span of several cellular divisions, leading to dysregulation of normal cellular functioning, and eventually malignant transformation (Hahn & Weinberg, 2002; Vogelstein et al, 2013). The genetic alterations can be somatic mutations (point mutations, few base-pair insertions and deletions) or somatic chromosomal aberrations such as aneuploidy, copy number alterations (CNAs) (deletion, amplification, inversion or translocation). On the other hand, epigenetic alterations can occur due to modulation of the chromatin structure (histone code), promoter DNA methylation pattern or other post-translational modifications leading to altered expression of a gene. Those alterations that provide a selective growth advantage to the tumor cells, and therefore drive tumorigenesis, are referred to as ‘driver mutations’, whereas, the rest are called ‘passenger mutations’ (Vogelstein et al, 2013). The type, frequency, and the sequence of driver alterations might differ depending on the cancer type. However, on an average a tumor harbors alterations in 2-8 driver genes and 30-60 passenger genes (Vogelstein et al, 2013). A total of 138 driver genes have been identified in cancers, which participate in 12 different signaling pathways regulating cell survival, proliferation, differentiation, DNA repair and other processes required for genome integrity (Vogelstein et al, 2013).

Conversely, genome instability arises from the acquired genetic and epigenetic alterations in a cell. This instability progressively generates a set of hallmark functional capabilities to malignant cells, which are required for tumor initiation and progression. However, similar hallmark capabilities can be acquired via distinct biological mechanisms in different cancer types. Six hallmark cancer capabilities were described by Hanahan and Weinberg in 2000, which were later updated with two new hallmarks in 2011 (Hanahan & Weinberg, 2000; 2011). These hallmark changes are briefly described here.

1) The 'sustained proliferative signaling' arising from the amplified autocrine or paracrine signaling via cell surface receptors, ligand-independent activation of the receptors or their downstream targets, or disruption of the negative-feedback signaling.

2) 'Insensitivity to growth suppressors' achieved by deletion or inactivation of tumor suppressors such as pRb (retinoblastoma protein or RB) and p53 (tumor protein 53 or TP53). If these proteins are active, then under stressful conditions they can promote cell-cycle arrest, senescence and even apoptotic cell death.

3) The 'evasion of the programmed cell death (apoptosis)' is required for the expansion of cancer cells to attain large tumor mass. It can be achieved by activating the intrinsic anti-apoptotic factors (e.g. BCL-2 or BCL-XL) or the upstream growth factor receptor survival signaling proteins (e.g. insulin growth factors IGF1/2). Alternatively, by inhibiting the pro-apoptotic factors (e.g. BAD, BAX, or PUMA) or the extrinsic death-receptor signaling proteins (e.g. Fas, TRAIL-R or TNFR).

4) The 'replicative immortalization' enables cancer cells to circumvent irreversible quiescence (senescence) and death (crisis phase). A normal cell encounters crisis phase due to telomere shortening that occurs after a certain number of replication cycles have completed. Upregulation of the telomerase reverse transcriptase (TERT) enzyme prevents the telomere erosion thereby making cancer cells immortal.

5) 'Sustained angiogenesis' promotes tumor growth by providing essential nutrients and oxygen consistent with the high metabolic activity of these cells. Angiogenesis is enhanced by the increased expression of angiogenic stimuli such as VEGF (vascular endothelial growth factor) or the inactivation of angiogenesis inhibitors (thrombospondin-1).

6) The 'activation of invasion and metastasis' is essential for the localized tumor cells to invade nearby tissue and metastasize to distant organs. It is acquired by altering the expression of cell-cell and cell-extracellular matrix (ECM) adhesion proteins (invasion-antagonist E-cadherin is downregulated whereas cell migration promoting N-cadherin is

upregulated), and/or by overexpressing the ECM degrading enzymes (matrix metalloproteases - MMPs).
7) 'Reprogramming of the energy metabolism' from the normal citric acid cycle to lactate producing glycolysis is essential for the cancer cell survival under hypoxic (low oxygen) microenvironment encountered in large tumors.

8) Cancer cells also device ways to 'avoid immune destruction' by a dynamic process referred to as 'immunoediting'. In a healthy human, natural killer (NK) cells or cytotoxic T-lymphocytes regularly detect and eliminate highly immunogenic cancer cells. This allows the selective growth and expansion of remaining weakly immunogenic cancer cell clones to generate tumors that are resistant to the immune attack.

The key molecular alterations that drive tumorigenesis in cultured human cells have been identified more than 15 years ago (Hahn et al, 1999; Hahn & Weinberg, 2002). It was demonstrated that the expression of hTERT and active Harvey-rat sarcoma viral oncogene homolog (H-Ras) oncogene along with simian virus 40 (SV40) large T-antigen (LT) and small t-antigen (ST) could transform normal human fibroblasts, embryonic kidney (HEK), and mammary epithelial cells (Hahn et al, 1999). Later it was shown that SV40-LT inhibits pRb and p53, whereas SV40-ST inhibits PP2A (Hahn et al, 2002; Pallas et al, 1990; Rangarajan et al, 2004). Thus, the list of minimum genetic alterations leading to human cell transformation have been assigned to these five proteins: active hTERT and H-Ras, and suppressed pRb, p53 and PP2A. However, some cell-type specific requirements for the activation of additional downstream factors of H-Ras such as Raf, PI3K or guanine nucleotide exchange factors (GEFs) have been observed (Rangarajan et al, 2004).

2.1.1.1. Apoptosis in normal and cancerous cells

This thesis explores the apoptosis induction in GBM cells in response to therapy, therefore this process is described here in more detail.

Apoptosis, a major form of 'programmed cell death', is an active cellular process that occurs in response to specific stimuli. Apoptosis results in characteristic morphological changes in a cell such as breakdown of cellular cytoskeleton, shrinkage of cellular components, membrane blebbing, nuclear fragmentation and encasement of cell contents into membrane bound vesicles called 'apoptotic bodies'. The apoptotic bodies are eventually engulfed by the neighboring phagocytic cells leaving no trace of a cell within few hours after the process of apoptosis had begun (Green & Llambi, 2015).

The apoptotic signaling can be triggered by ligand binding to cell surface death receptors (extrinsic pathway) or by activation of B-cell lymphoma

2 (Bcl-2)-family pro-apoptotic proteins that lead to permeabilization of mitochondrial membrane (intrinsic pathway). Both pathways converge on the activation of caspase proteases, which via a cascade of events result in the cleavage of cellular proteins and ultimately apoptosis (Green & Llambi, 2015). The caspases are broadly categorized into two groups, the initiator caspases (caspase-2, 8, 9 and 10), and the effector caspases (caspase-3, 6 and 7). The initiator caspases become activated upon binding with the caspase-activation platforms containing death domains (Green & Llambi, 2015). These platforms often serve as a site for the recruitment of initiator caspases, bringing caspase monomers in close proximity for autocleavage and activation (Green & Llambi, 2015). The activated initiator caspases further cleave the inactive procaspase-3 and -7, generating active effector caspase-3 and -7, which in turn cleave procaspase-6 and thousands of other cellular proteins (Fischer et al, 2003; Green & Llambi, 2015). The cleavage of some cellular proteins results in their inactivation and disruption of their function, for example cleavage of Lamin A leads to disassembly of the nuclear membrane (Fischer et al, 2003; Green & Llambi, 2015). Whereas some proteins become activated upon caspase cleavage, such as Gelsolin cleavage leads to constitutive activation of its actin depolymerization function, resulting in cytoskeleton destruction and membrane blebbing (Fischer et al, 2003; Green & Llambi, 2015).

The extrinsic pathway can be invoked by the binding of ligands to the cell surface death receptors (DRs). For instance, TNFα released by immune cells can bind to the TNF-R1 receptor expressed on the surface of some transformed cells, and induce apoptosis in these cells (Green & Llambi, 2015). CD95 or Fas and TRAIL-R are other two important death receptors. The extrinsic apoptotic signaling often results in the activation of caspase-8 or -10, which further cleaves and activates the effector caspases to induce apoptosis (Green & Llambi, 2015).

The intrinsic pathway is triggered by various stress signals, such as DNA damage (including that induced by chemotherapy or irradiation), endoplasmic reticulum (ER) stress, accumulation of unfolded proteins, or nutrient deprivation (Czabotar et al, 2014; Green & Llambi, 2015). The Bcl-2-family proteins orchestrate the intrinsic apoptosis pathway by regulating the mitochondrial outer membrane permeabilization (MOMP) (Czabotar et al, 2014; Green & Llambi, 2015). All Bcl-2-family proteins contain one or more Bcl-2 homology (BH) domains, and based on their function are categorized into three types, the pro-apoptotic effector proteins (BAK and BAX), the anti-apoptotic proteins (BCL-2, BCL-XL and MCL1), and the pro-apoptotic BH3-only derepressor proteins (BAD, BID, BIM, PUMA and NOXA).

Under normal conditions, anti-apoptotic BCL-2, BCL-XL and MCL1 remain bound to the pro-apoptotic effectors (BAK and BAX) located in

the outer membrane of mitochondria (Czabotar et al, 2014; Green & Llambi, 2015). This binding inhibits the pro-apoptotic function of BAK and BAX. However, upon receiving the stress signals, the pro-apoptotic proteins BAD, BID, PUMA, or NOXA translocate from cytoplasm to the mitochondria, and bind to the anti-apoptotic proteins (Czabotar et al, 2014; Green & Llambi, 2015). This relives the pro-apoptotic effectors BAK and BAX from anti-apoptotic proteins, and they start to form large oligomers. These oligomers get inserted into the outer mitochondrial membrane, creating pores and enhancing permeabilization (MOMP) (Czabotar et al, 2014; Green & Llambi, 2015). The MOMP results in the release of cytochrome *c* into the cytoplasm, which binds to the apoptotic protease-activating factor 1 (APAF1) to create an oligomeric complex (caspase-activating platform) called 'apoptosome' (Czabotar et al, 2014; Green & Llambi, 2015). The apoptosome recruits caspase-9 monomers and promotes their autocleavage and activation. This results in the activation of caspase-3, 6 and 7 as described above.

Interestingly, different BH3-only pro-apoptotic derepressor proteins act as a node between different types of stress signals and intrinsic apoptosis (Czabotar et al, 2014; Green & Llambi, 2015). For instance, several growth factor signaling pathway targets (e.g. PI3K/AKT/p70-S6K, MAPK/ERK/p90-RSK and PKA) promote BAD phosphorylation and inhibition of its pro-apoptotic function (Danial, 2009; Lindsay et al, 2011). Therefore, the inhibition of these survival pathways and/or direct BAD dephosphorylation can potentially lead to apoptosis via intrinsic apoptosis pathway. On the other hand, DNA damage induced apoptosis is transduced by PUMA and NOXA (Chaabane et al, 2013; Green & Llambi, 2015). Persistent DNA damage activates p53, which transactivates the expression of PUMA and NOXA, followed by the cascade of events described above resulting in apoptosis. Interestingly, the hyperactivation of oncogenic Myc can also lead to apoptosis in a p53 dependent manner (Green & Llambi, 2015; Hanahan & Weinberg, 2011). Therefore, loss of function mutations or deletions of *TP53* help evade the death of malignant cells, that may have followed in response to accumulating DNA damage lesions (Hanahan & Weinberg, 2011). Another way for cancer cells to evade apoptosis is by shifting the balance between pro- and anti-apoptotic Bcl-2-family proteins (Hanahan & Weinberg, 2011). It can happen by overexpression or stabilization of anti-apoptotic proteins, or by inhibition of pro-apoptotic proteins. For instance, BCL-2 overexpression cooperates in the Myc driven tumorigenesis in lymphoma mouse models (Czabotar et al, 2014). The aberrantly active PI3K/AKT or Ras/MAPK/ERK pro-survival signaling present in many solid tumors including glioblastoma, breast or prostate cancers results in phosphorylation and inhibition of pro-apoptotic protein BAD (Danial, 2009). In order to shift the Bcl-2-family protein balance to the pro-apoptotic side in cancer cells, some BH3-mimetic

compounds have been developed (Czabotar et al, 2014). These compounds, such as ABT263 resemble BH3-only proteins (e.g. BAD) in their structure, and carry out analogous function of inhibiting the BCL-2 and BCL-XL anti-apoptotic proteins (Czabotar et al, 2014). Some of these apoptosis inducing BH3-mimetic compounds have been under investigation as anti-cancer agents (Czabotar et al, 2014).

2.2. Brain tumors

The tumors of the brain and spinal cord tissue of central nervous system (CNS) collectively accounted for 1.8% of all diagnosed cancers worldwide in 2012 (Ferlay et al, 2015). During that year, 189000 brain tumor related deaths were recorded (2.3% of the total cancer related deaths) (Ferlay et al, 2015). Brain tumors can be classified based on the primary tumor site and the resemblance of tumor cells to different types of adult non-neoplastic brain cells (Louis et al, 2007; Ricard et al, 2012). The two most common classes of primary brain tumors are glioma (tumors of neuroepithelial tissue) and meningioma (tumors of meningeal tissue) that comprise 70% and 27% respectively of total primary brain tumor burden (Louis et al, 2007; NCI PDQ, 2016; PDQ, 2002; Ricard et al, 2012). Gliomas are further separated into three main histological sub-types: astrocytomas, oligodendrogliomas and oligoastrocytomas (Louis et al, 2007; Ricard et al, 2012).

Gliomas can originate from undifferentiated or dedifferentiated cells that resemble neural stem or progenitor cells in many ways, referred to as glioma stem cell (GSC) (Ricard et al, 2012) (discussed later in this review) (Figure-1). Tumor cell proliferation, differentiation, mitotic activity, atypia, microvascular proliferation and necrosis are used to define the WHO grade of gliomas (Louis et al, 2007; Ricard et al, 2012). Those tumors that do not show tissue infiltration and are curable by surgery alone, such as pilocytic astrocytomas, are classified as WHO grade I. The astrocytic glioma tumors with moderate cyto-nuclear atypia and diffuse tissue infiltration are referred to as grade II (low-grade diffuse astrocytoma), those diffuse astrocytomas which are poorly differentiated (anaplastic) and show higher mitotic activity as grade III (anaplastic astrocytoma or AA), and those with additional presence of microvascular proliferation and necrosis as grade IV (glioblastoma or GBM) (Louis et al, 2007; Ricard et al, 2012). The oligodendrogliomas and mixed oligoastrocytomas are also classified as grade II (low-grade) or grade III (anaplastic) (Louis et al, 2007; Ricard et al, 2012). All three lineages of low-grade gliomas (LGGs) can progress into anaplastic gliomas (grade III) and finally glioblastoma (GBM), referred to as secondary GBM (Figure-1) (Louis et al, 2007; Ohgaki & Kleihues, 2013; Ricard et al, 2012).

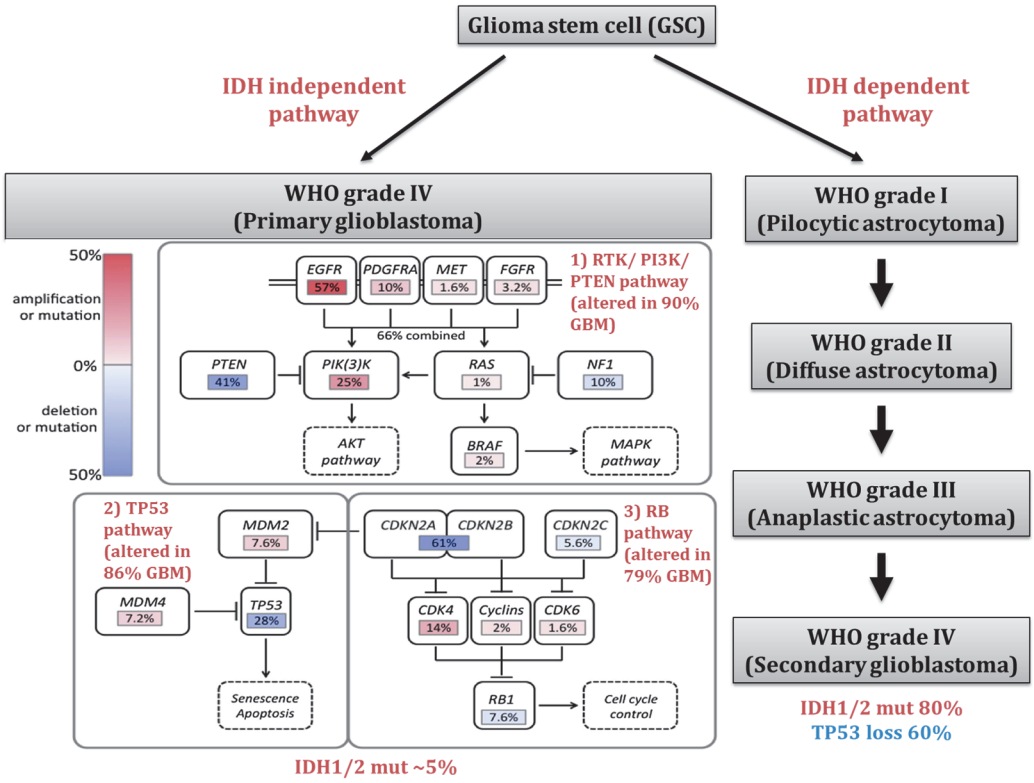


Figure-1 Generation of gliomas and key molecular alterations associated with GBM. *Primary and secondary gliomas originate by pathways independent or dependent on IDH1 or IDH2 mutations, and differ in the frequency of TP53 alterations. Secondary GBM may also originate from oligodendroglioma or oligoastrocytoma (not shown) in addition to astrocytoma. Red boxes or text indicate alterations that lead to activation, and blue indicate the inactivation of altered genes (modified from (Brennan et al, 2013)).*

However, *de novo* highly aggressive glioblastoma cases, referred to as primary glioblastomas, are more frequently detected in the clinic (>90% of total GBM cases) (Figure-1) (Louis et al, 2007; Ohgaki & Kleihues, 2013; Ricard et al, 2012). The histological type and grade of gliomas are strong prognostic indicators of patient survival (Louis et al, 2007; Ricard et al, 2012). Patients with oligodendroglioma have better survival than with oligoastrocytoma, which in turn perform better than those with astrocytic gliomas. The correlation of increasing glioma tumor grade (aggressiveness) with poor patient prognosis is very dramatic. The median overall survival (OS) of patients with LGGs is 6-12 years, with anaplastic gliomas 3-10 years (3 years for AA), and with primary or secondary GBM only 1-2 years (Louis et al, 2007; Ricard et al, 2012). The frequency of detection of LGG and anaplastic gliomas is 25% each,

whereas GBM accounts for the remaining majority (50%) of adult glioma cases (Ricard et al, 2012). LGGs are usually detected in young adults (30-45 years age), anaplastic glioma in individuals aged around 45 years, and GBM in elderly patients (60 years) (Ricard et al, 2012).

2.2.1. Glioblastoma (GBM)

GBM is the most aggressive, and frequently detected adult glioma, associated with absolute mortality. The first line of treatment for GBM is maximum surgical tumor resection and adjuvant conventional radiotherapy and/or chemotherapy. The advanced intraoperative tumor visualization tools available nowadays have allowed the surgeons to achieve very high surgical resection, however, without substantial improvement in the patient survival (Kuhnt et al, 2011). The adjuvant therapy with combined radiation and temozolomide (TMZ), which is the best available and current standard treatment for GBM, extends the median OS to only 14.6 months and 5-year survival rate of less than 10% (Stupp et al, 2009; Stupp et al, 2005). The reasons behind the ineffective treatment for GBM are: 1) the characteristic infiltrative/ invasive tumor growth making it impossible to completely resect the tumors (surgically incurable), 2) inherent resistance to conventional therapies, and 3) tumor heterogeneity (Cloughesy et al, 2014; Furnari et al, 2007; Meyer et al, 2015). A lot of research efforts during the past decade have been focused on the molecular characterization and subtyping of GBM, which has led to a better understanding of this fatal disease.

2.2.1.1. Molecular alterations in glioblastoma

The very initial attempt to study genetic alterations in malignant gliomas was reported in 1985. Libermann et al described the amplification and genetic rearrangement of oncogene *EGFR* (epidermal growth factor receptor) responsible for overexpression of a full length and a truncated EGFR (ΔEGFR or EGFRvIII) in human GBM (Libermann et al, 1985a; b). In the following years these results were confirmed by independent research groups, with some suggesting a link between patient prognosis and *EGFR* amplification and/or overexpression (Bigner et al, 1988; Ekstrand et al, 1992; Humphrey et al, 1991; Hurtt et al, 1992; Malden et al, 1988; Yamazaki et al, 1988). Around the same time, loss of chromosome 17p, which contains *TP53* gene, and loss of other chromosomes (1p, 9p, 10, 19q and 22) were found in glioma and other brain tumors (Bigner & Vogelstein, 1990; Louis & Gusella, 1995; Mashiyama et al, 1991). These pioneer studies also indicated that glioma subsets with distinct molecular profile might exist which could not be separated solely on the basis of histological and clinical parameters, and a better understanding of the molecular changes might direct the way to

better brain tumor diagnosis and treatment (Bigner et al, 1988; Louis & Gusella, 1995; Rey et al, 1992). Later on, with the advent of next generation sequencing, gene expression arrays, tissue microarrays (TMA), and bioinformatics analysis tools, the path to molecular profiling of human gliomas started to get uncovered (Fuller et al, 2002; Mischel et al, 2003; Sallinen et al, 2000).

Molecular alterations in gliomas affect key signaling pathways and are tightly linked with their hallmark biological features, such as increased cell proliferation, defective cell cycle control, inherent resistance to apoptosis (by conventional therapies), microvascular proliferation and angiogenesis, enhanced necrosis, and extensive tissue invasion (Cloughesy et al, 2014; Furnari et al, 2007). A comprehensive knowledge of these molecular changes came from the cancer genome atlas (TCGA) guided genomic and transcriptomic profiling analysis of a multi-national human glioma tumor cohort (TCGA, 2008). In addition to validating the findings of a number of previous reports, TCGA identified novel GBM related alterations which cluster into three highly interconnected core pathways: 1) RTK/PI3K/PTEN signaling, 2) p53 signaling, and 3) pRb signaling, showing alterations in 90%, 86% and 79% of GBMs respectively (TCGA, 2008) (Figure-1). During the same year, using an unbiased high-density genomic and integrated transcriptomic analysis, another research group not only confirmed similar core pathway alterations, but also discovered mutations in *IDH1* (isocitrate dehydrogenase 1) as major determinant of a novel GBM subtype associated with dramatically better patient survival (Parsons et al, 2008). In 2013, TCGA consortium released an updated and more comprehensive analysis of the GBM cohort containing more than 500 primary GBM samples (Brennan et al, 2013).

Among the RTK pathway, amplification or gain-of-function (GOF) somatic mutations in *EGFR* (57%), platelet-derived growth factor receptor A (*PDGFRA*) (10%), *ERBB2* (8%), *PIK3CA* (15%), and homozygous deletions or loss-of-function (LOF) mutations in *PTEN* (phosphatase and tensin homolog) (41%) and *NF1* (10%) are frequently detected, leading to increased activity of this pro-survival signaling pathway in GBM (Brennan et al, 2013; Parsons et al, 2008; TCGA, 2008) (Figure-1). The tumor suppressor p53 and pRb signaling pathways are inhibited by LOF mutations or deletions of *TP53* (28%), cyclin-dependent kinase inhibitors (CDKN): *CDKN2A* (58%), *CDKN2B* (47%), and *RB1* (8%), as well as by amplification of cyclin-dependent kinases (CDK): *CDK4/CDK6* (15%), mouse double minute 2 homolog (MDM): *MDM2* (8%) and *MDM4* (7%), altogether resulting in altered cell cycle progression and evasion of cell death (Parsons et al, 2008; TCGA, 2008) (Figure-1). A great majority (74%) of GBMs harbor alterations in all three pathways, although a mutual exclusivity of alteration of members of the same

pathway also exists (Parsons et al, 2008; TCGA, 2008). Alterations in *IDH1* were initially detected in 12% of all GBMs, with somatic point mutations almost exclusively resulting in single amino acid substitution at Arg132 (R132H) (Parsons et al, 2008). Later on, mutations in *IDH1* or its relative *IDH2* were found in a nearly 80% of grade II and III gliomas and secondary GBMs, and a rather small fraction (~5%) of primary GBMs (Brennan et al, 2013; Ohgaki & Kleihues, 2013; Yan et al, 2009) (Figure-1). Therefore, it is now established that the secondary GBMs originate via IDH-dependent pathway, whereas the primary GBMs arise in IDH-independent manner (Figure-1). IDH1 is a metabolic enzyme, and its mutation at R132 alters the substrate specificity, resulting in the production of an oncometabolite 2-hyroxyglutarate (2-HG), which has widespread effects on cancer cell metabolism, oxidative stress, histone methylation and gene transcription (Cloughesy et al, 2014; Dang et al, 2009).

The DNA methylation pattern analysis of TCGA GBM cohort led to the identification of a distinct DNA hypermethylation cluster, referred to as glioma CpG island methylator phenotype (G-CIMP) (Noushmehr et al, 2010). Intriguingly, the mutant IDH1 oncometabolite 2-HG inhibits histone demethylases resulting in increased histone H3 methylation (at K27, K9 or K36 residues), and subsequent DNA methylation pattern similar to G-CIMP (Turcan et al, 2012). Accordingly, virtually all IDH1-mutant gliomas are associated with the presence of G-CIMP signature (Parsons et al, 2008; Turcan et al, 2012). Lastly, another core pathway containing histone H3.3 (*H3F3A*), ATRX and DAXX involved in chromatin remodeling was found to be altered in 44% of pediatric GBMs (Schwartzentruber et al, 2012). The *H3F3A* mutations (31%) were responsible for amino acid substitutions resulting in either K27M or G34R/V mutant H3.3. Interestingly, the *IDH1* and *H3F3A* mutations were mutually exclusive in these GBM samples (Schwartzentruber et al, 2012).

2.2.1.2. Molecular classification of glioblastoma

Earlier studies by independent research groups have identified the existence of distinct genomic and transcriptomic alterations associated with histologically indistinguishable primary and secondary GBMs, which facilitated the investigation of further molecular subtyping of GBM to distinct classes with possibly different prognosis, and predictive response to targeted treatments (Maher et al, 2006; Ohgaki & Kleihues, 2013; Phillips et al, 2006; Shai et al, 2003). Verhaak et al have performed the hierarchical clustering of transcriptional profiles integrated with genetic alterations (CNAs) and sequencing (mutational) data from a large panel of adult GBMs (TCGA and independent cohorts), to reveal four molecularly distinct GBM subclasses (Verhaak et al, 2010). These

include: 1) 'Classical' subtype characterized by *EGFR* amplification (including point mutations and EGFRvIII) and *CDKN2A* deletion; 2) 'Mesenchymal' subtype with deletion or mutation directed inhibition of *NF1*, *TP53*, and *CDKN2A*; 3) 'Proneural' subtype with *PDGFRA* amplification and *IDH1* mutation as major alterations, but, also mutations or loss of *TP53*, amplifications of *CDK4*, *CDK6* and *MET*, mutations of *PIK3A/PIKR1* and high *OLIG2* expression; 4) 'Neural' subtype did not display any characteristic alterations apart from the expression of neuronal markers and close resemblance with the normal brain tissue profile (Verhaak et al, 2010). By further addition of the epigenetic alterations as another layer, the genome-wide DNA methylation analysis and clustering have resulted in the separation of *IDH1*-mutated and G-CIMP positive subtype from the proneural group (Noushmehr et al, 2010). Finally, Sturm et al have combined pediatric GBMs along with the adult GBMs to perform integrated epigenetic, genetic and transcriptional analysis to define six GBM subgroups, which not only display distinct molecular profile but also show difference in age distribution, tumor location and patient prognosis (Sturm et al, 2012). These GBM subtypes are: IDH, K27, G34, RTK-I PDGFRA, RTK-II classical, and mesenchymal. The IDH subtype is characterized by mutations in *IDH1* and *TP53,* and G-CIMP positivity. The K27 and G34 subtypes are characterized by mutations at respective amino acid residues in histone H3.3 (*H3F3A)*, as well as *TP53* mutations (Sturm et al, 2012). RTK-I subtype contains *PDGFR* amplification and *CDKN2A* deletion (Sturm et al, 2012). The IDH, K27 and RTK-I subtypes resemble Verhaak's 'Proneural' transcriptional profile (Sturm et al, 2012; Verhaak et al, 2010). The RTK-II subtype is essentially similar to 'Classical' subtype and the mesenchymal similar to 'Mesenchymal' subtype from Verhaak's classification system (Sturm et al, 2012; Verhaak et al, 2010). Brennan et al have performed similar integrated profiling from mainly adult primary GBM samples (TCGA dataset) (Brennan et al, 2013). They have proposed six subclasses (M1, M2, M3, M4, G-CIMP, and M6) that partially overlap with the previously reported classifications (Brennan et al, 2013; Sturm et al, 2012). However, due to the lack of *H3F3A* mutations in adult GBM, the K27 and G34 subclasses reported by Sturm et al could not be assigned to any subclass reported by Brennan et al, highlighting the specific role of these mutations in pediatric GBMs (Brennan et al, 2013; Sturm et al, 2012).

2.2.1.3. Therapy resistance in glioblastoma

The major underlying cause of therapy resistance in GBM (in fact cancers in general) is the tumor heterogeneity, which exists due to differences in the individual patients (personalized), differences in separate tumors within a patient (inter-tumor), and differences in the cells within a tumor (intra-tumor).

The intratumoral heterogeneity is an inherent property of GBM, and it can arise from the non-mutually exclusive existence of cellular hierarchies, genetic and/or epigenetic variability, and different tumor microenvironment (Cloughesy et al, 2014; Furnari et al, 2015). The cancer stem cell model is an example of the tumors with differentiation state hierarchy; wherein, a small subset of undifferentiated, stem-cell-like tumor cells (in this context referred to as glioma stem cells – GSCs) give rise to both GSC and differentiated cell progeny (Pointer et al, 2014). The GSCs display immense resistance to drug and radiation therapies and extracellular stress conditions such as nutrient and oxygen deprivation, possibly due to different epi(genetic) makeup and signaling networks, and lower rate of cell proliferation (Chen et al, 2012; Patel et al, 2014; Singh et al, 2004). Therefore, GSCs can invariably lead to tumor regrowth in a setting where initial treatment-associated tumor regression was noticed due to the death of treatment-sensitive differentiated tumor cells. The GSC model is however partially opposed by the dedifferentiation theory. Studies in glioma animal models demonstrated that introduction of a specific set of genomic alterations can promote malignant transformation not only in neural stem cells but also in astrocytes and neurons (differentiated cells), providing a counterargument that the altered oncogenic signaling can induce dedifferentiation to generate *de novo* GSCs (or GSC-like cells) responsible for tumor initiation, maintenance and intratumoral heterogeneity (Bachoo et al, 2002; Cloughesy et al, 2014; Friedmann-Morvinski et al, 2012).

The clonal evolution and selection of 'driver' lesions sustain tumor cell subpopulations with considerable genetic and epigenetic heterogeneity within a tumor (regardless of their stem-cell state) as revealed by single-cell DNA and RNA sequencing analysis (Meyer et al, 2015; Patel et al, 2014). This model implies that the drug-resistant tumor clones pre-exist in variable abundance in primary tumor, which sustain tumor growth when treatment is directed towards one (most likely dominant) clone in that tumor. Secondly, upon treatment, further clonal selection in GBM leads to tumor recurrence by following evolutionary paths that can be either linear (recurrence descends from dominant clone in primary tumor) or branched (recurrent clone derived from a secondary branch diverged at an early evolutionary stage from the dominant primary tumor clone) (Johnson et al, 2014; Ramaswamy & Taylor, 2015). Therefore, clonal evolution acts as a major contributor to GBM resistance to targeted therapies, especially those that are based on the molecular characteristics of primary tumor. Strikingly, the treatment itself can induce clonal evolution and tumor progression (Johnson et al, 2014; Ramaswamy & Taylor, 2015). DNA damaging chemotherapy drug TMZ has been repeatedly shown to promote a hypermutator phenotype in recurrent gliomas and progression of LGGs to HGGs (Johnson et al,

2014; Parsons et al, 2008; TCGA, 2008). The clonal selection model is reinforced by the active intercellular signaling mechanisms which allow the tumor cells of one lineage to promote the growth of other lineages within a tumor (clonal cooperation) to sustain tumor heterogeneity (Furnari et al, 2015). For instance, the mutant EGFRvIII-expressing glioma cells utilize IL-6 and LIF cytokine-mediated paracrine signaling to promote proliferation of EGFR-expressing tumor cells, and the overall GBM tumor growth (Inda et al, 2010).

Additional layer of tumor heterogeneity and therapy resistance comes from the tumor microenvironment. Tumors originating at different locations in the brain appear to display different molecular signatures (Sturm et al, 2012). The hypoxic, nutrient-deprived microenvironment and an active intercellular communication via secreted growth factors and RTK signaling or ECM proteins profoundly influence the tumor growth (Cloughesy et al, 2014). The presence of aberrant tumor vasculature is a hallmark of GBM that has been historically associated with the formation of new blood vessels by endothelial cells (angiogenesis). Angiogenesis in tumors is mainly mediated by a cytokine VEGF (ligand) secreted by tumor cells to induce VEGFR (receptor) signaling in nearby endothelial cells, resulting in the formation of new blood vessels for providing nutrient and oxygen to facilitate tumor growth (Hardee & Zagzag, 2012). Recently, it was found that in addition to angiogenesis and vasculogenesis modes of new blood vessel formation from endothelial or progenitor cells respectively, the GSCs or other tumor cells can also differentiate into tumor-endothelial cells by a process called transdifferentiation (Hardee & Zagzag, 2012). Additionally, GBM displays properties of vascular co-option (tumor growth preferentially around existing blood vessels), and vascular mimicry to sustain its growth (Hardee & Zagzag, 2012). Vascular co-option and transdifferentiation are particularly important in driving resistance to anti-angiogenic therapies because of their inertness to these inhibitors (Cloughesy et al, 2014; Hardee & Zagzag, 2012). Moreover, the blood vasculature in brain constitutes a physical barrier, called blood-brain barrier (BBB) that restricts the entry of many therapeutic compounds to brain tissues. Thus BBB also contributes to drug resistance (discussed later in this review).

The most intensively tested molecular therapy target in GBM is EGFR. A series of receptor tyrosine kinase inhibitors (TKis) against EGFR (EGFRi) and related RTKs have been tested in the clinical trials, however, with very dismal improvement in the patient survival. In addition to the above-mentioned pathophysiological features of gliomas, specific molecular/pathway alterations have been linked with EGFRi resistance. EGFR activation functions by triggering the downstream PI3K/AKT/mTOR signaling pathways. Tyrosine phosphatase PTEN is a

major negative regulator of this pathway and it functions by reversing the action of PI3K-mediated AKT phosphorylation. Therefore, loss of *PTEN* (found in >40% GBMs) confers resistance to EGFRi by maintaining a continued PI3K pathway activity in tumor cells (Cloughesy et al, 2014; Furnari et al, 2015). The EGFRi resistance is further enhanced by the expression of ligand-binding domain mutant EGFR variant, EGFRvIII, which acts as a constitutive and more potent inducer of PI3K signaling than wild-type EGFR (Cloughesy et al, 2014; Furnari et al, 2015). Additional mechanisms such as clonal selection of other amplified RTKs (e.g. *PGDFRA*, *ERBB2*), or derepression of the RTKs (e.g. PDGFRB) which were previously suppressed by EGFR-signaling (feedback activation) or the reversible loss of extrachromosomal amplified DNA (known for EGFRvIII) can promote resistance to EGFR inhibitors (Cloughesy et al, 2014; Furnari et al, 2015). A similar feedback activation of the PI3K-AKT and MAPK pathways and associated drug resistance is observed in GBM patients treated with mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin (Cloughesy et al, 2014; Mellinghoff et al, 2012). A mismatch between the inhibitor and the dominant mutation present in targeted tumors, and/or the insufficient drug delivery to the brain are other confounding factors responsible for drug resistance in GBM (Cloughesy et al, 2014).

Apart from the above-described therapy resistance mechanisms, gliomas also display inherent resistance to apoptosis, especially to that mediated by extrinsic apoptosis pathways. The underlying mechanisms of this apoptosis resistance are not completely known. However, the constitutively active RTKs and downstream MEK/ERK and PI3K pathway signaling has been attributed to desensitization of gliomas to apoptosis (Krakstad & Chekenya, 2010; Wojton et al, 2016). Specifically, EGFR and EGFRvIII expression confers apoptosis resistance in gliomas by inhibiting pro-apoptotic protein PUMA, and by enhancing the expression of anti-apoptotic BCL-xL (Wojton et al, 2016). Additionally, defects in the apoptosis pathways downstream of the death-receptors and even downstream of caspases may result in apoptosis resistance in gliomas. For instance, apoptosis inhibitory proteins such as XIAP (X-linked inhibitor of apoptosis) are highly expressed in malignant gliomas, and their expression relates to worse prognosis (Krakstad & Chekenya, 2010; Wagenknecht et al, 1999). Their expression results in blockage of apoptosis induction by both intrinsic and extrinsic apoptosis pathways (Krakstad & Chekenya, 2010). Interestingly, stimulation of death receptor Fas in glioma cells leads to activation of ERK-signaling, cell cycle progression and cell proliferation that surpasses the cell death mediated by extrinsic apoptosis, thereby resulting in apoptosis resistance (Shinohara et al, 2000). Thus, Fas/ERK pathway might function as an autocrine growth factor signaling mechanism in glioma tumors that simultaneously express Fas and Fas-L, and especially those

in which apoptosis is inhibited by defects in caspase-8 or its downstream effectors (Saggioro et al, 2014; Shinohara et al, 2000). Notably, the activation of intrinsic apoptosis pathway and activation of caspase-3 results in cell death in the glioma cells which are resistant to FasL-induced extrinsic apoptosis (Karlsson et al, 2004).

2.2.1.4. Kinase inhibitor therapies in glioblastoma

Since RTK/PI3K signaling pathway is frequently altered in GBM, it is an obvious choice for molecularly targeted therapies. Moreover, RTKs and its downstream kinases are 'druggable' targets, and can be inhibited by small molecule inhibitors, ligand-neutralizing or receptor blocking antibodies or peptides. Table-1 highlights various kinase-targeted therapies and their status in clinical trials for GBM and other malignant gliomas.

Among RTKs, the EGFR and its mutant variant EGFRvIII have been most prominent drug targets in GBM. Small molecule inhibitory compounds targeting EGFR are classified as first-generation reversible inhibitors (erlotinib, gefitinib, and lapatinib) or second-generation irreversible inhibitors (afatinib and dacomitinib). The first-generation EGFRis fail to achieve adequate CNS and intratumoral drug concentration in GBM patients, and are often associated with inefficient inhibition of downstream signaling (drug resistance mechanisms described earlier) (Cloughesy et al, 2014; Reardon et al, 2014). Moreover, erlotinib and gefitinib are pharmacologically less efficient in binding to the inactive conformation of EGFRvIII expressed from the extrachromosomal DNA (double minutes) in GBM, (Cloughesy et al, 2014). The second-generation EGFRis are expected to perform better in these settings, and are therefore under clinical trials (Reardon et al, 2015; Reardon et al, 2014). The resistance to EGFRi can occur due to co-activation of other RTKs, which can be alleviated by inhibitors that simultaneously inhibit RTKs apart from EGFR. For instance, lapatinib and afatinib inhibit other EGFR-family RTKs, ERBB2/4, and vandetanib inhibits VEGFR2 in addition to EGFR (Cloughesy et al, 2014; Reardon et al, 2014). Several other multi-target TKis with different kinase-inhibition profile have been tested in glioma clinical trials, with several of these targeting RTKs such as PDGFR, MET and FGFR, responsible for compensatory activation of GBM growth and survival (Table-1). The VEGFR inhibitors on the other hand function by suppressing the tumor-associated angiogenesis therefore inhibiting the tumor growth by restricting nutrient supply (Bai et al, 2011; Hardee & Zagzag, 2012). Alternatively, the tumor vasculature can be normalized by VEGF-neutralizing antibodies or decoy-receptor fusion proteins such as bevacizumab and aflibercept respectively (Bai et al, 2011; Lau et al, 2014; Weller et al, 2013). In fact, the humanized monoclonal anti-VEGF antibody, bevacizumab, has

gained the United States food and drug administration (FDA) approval as second line treatment for recurrent GBM, although only a modest increase in progression free survival (PFS) was noticed in the initial clinical trials, with no overall survival (OS) or health-related quality of life (HRQoF) benefits in repeated single-agent or combination studies (Cohen et al, 2009; Reardon et al, 2015; Taphoorn et al, 2015).

Additional approaches combining EGFRi with inhibition of downstream targets PI3K or mTOR has been proposed to counteract drug resistance. There are two types of mTOR inhibitors: mTORC1 inhibitor rapamycin analogs or rapalogs (temsirolimus and everolimus), and ATP-competitive mTORC1/2 dual inhibitors (Cloughesy et al, 2014). The rapalog combination with EGFRis has displayed only a limited anti-glioma activity, which is often masked by the moderate to high toxicity in patients (Wen et al, 2014). Rapalogs are also subjected to feedback activation of PI3K and mTORC2 signaling leading to drug resistance (Cloughesy et al, 2014; De Witt Hamer, 2010; Lau et al, 2014). Phase I/II trials evaluating the tolerated dose of dual mTORC1/2 inhibitors, AZD2014 and MLN0128, are ongoing (www.clinicaltrials.gov). The dual inhibitors should be less prone to the drug resistance mechanisms faced by rapalogs.

Among other serine/threonine kinases, inhibition of protein kinase C (mainly PKCβ) by small molecule enzastaurin has been evaluated in GBM. Despite a good BBB-penetration and well-tolerated toxicity profile, enzastaurin displayed only limited efficacy in clinical trials in recurrent or newly diagnosed GBM patients (Lau et al, 2014; Wick et al, 2010; Wick et al, 2013). A novel class of Ras inhibitor Lonafarnib (Sarasar, SCH66336), which inhibits farnesyl transferase enzyme required for Ras activation, was well tolerated in combination with TMZ in a phase I trial (Lau et al, 2014).

Despite several efforts, no significant increase in GBM patient survival has been found so far. The disappointing results are related to tumor heterogeneity, drug resistance pathways and poor pharmacokinetics (including BBB-penetration). Several immunotherapy approaches for GBM are currently being pursued in the quest for therapies that could extend patient survival. In this context, a peptide corresponding to EGFRvIII, rindopepimut, acts as a therapeutic vaccine in stimulating the patient's immune cells to target and eliminate EGFRvIII-expressing tumor cells. Strikingly, rindopepimut treatment significantly extends GBM patient survival (median OS 21.8 months) with minimal toxicity (Schuster et al, 2015). Therefore, this strategy has opened new avenues in the treatment possibilities for this deadly disease.

Table-1: ***Kinase inhibitor therapies in glioma clinical trials.***

Drug	Target	Clinical trial	Complications	Ref
Small molecule inhibitors				
Erlotinib	EGFR	Phase II; ongoing	Insufficient delivery (poor CNS and intratumoral penetration); resistance	1; 5
Gefitinib	EGFR	Phase II	Limited BBB-penetration; resistance	1; 5
Lapatinib	EGFR, ERBB2	Phase I/II; ongoing	Resistance; inefficacy	1; 5
Afatinib	EGFR, ERBB2, ERBB4	Phase I/II; ongoing	Limited efficacy	6
Vandetanib	EGFR, VEGFR2	Phase I/II	Inefficacy; toxicity (seizures)	1; 5
Cediranib	VEGFR	Phase III	Drug efflux by BBB transporters; inefficacy	2
Cabozantinib (XL184)	VEGFR, MET, RET, KIT	Phase II	Toxicity	2
Pazopanib	VEGFR, KIT, PDGFR	Phase II; ongoing	Inefficacy	4; 7
Imatinib	KIT, RET, BCR-ABL	Phase III	Poor BBB penetration; inefficacy	3; 4
Sunitinib	PDGFR, KIT VEGFR, FLT3, RET	Phase II	Drug efflux by BBB transporters; inefficacy	3; 4
Sorafenib	Raf, PDGFR, KIT, VEGFR, FGFR, FLT1/3, RET	Phase II; ongoing	Limited efficacy in combination with TMZ	3; 4
Galunisertib (LY2157299)	TGFβR1	Phase II	Inefficacy in combination with lomustine	8
Temsirolimus (CCI-779)	mTORC1	Phase II; ongoing	High toxicity; inefficacy in combination with erlotinib	4; 5
Everolimus (RAD001)	mTORC1	Phase I/II; ongoing	Moderate-high toxicity; inefficacy	5; 10
Enzastaurin	PKCβ,α,γ,ε	Phase II/III	Limited efficacy	3; 4
Monoclonal antibodies				
Cetuximab	EGFR	Phase II; ongoing	Limited BBB-penetration; inefficacy in unselected patients	1; 5
Nimotuzumab	EGFR	Phase III	Limited efficacy; unrelated to *EGFR* amplification	5; 9
Bevacizumab (Avastin)	VEGF-A	Approved; Phase III; ongoing	No improvement in patient's OS or HRQoL	2; 11
Recombinant fusion proteins or peptides				
Aflibercept (VEGF trap)	VEGF, PGF	Phase II	Moderate toxicity; limited efficacy	2; 5
Rindopepimut (CDX-110)	EGFRvIII	Phase II; ongoing		12

References: 1. (Reardon et al, 2014); 2. (Weller et al, 2013); 3. (Lau et al, 2014); 4. (De Witt Hamer, 2010); 5. (Bai et al, 2011); 6. (Reardon et al, 2015); 7. (Iwamoto et al, 2010); 8. (Brandes et al, 2016); 9. (Westphal et al, 2015); 10. (Ma et al, 2015); 11. (Taphoorn et al, 2015); 12. (Schuster et al, 2015)

2.2.1.5. Blood-brain barrier (BBB)

Human brain is one of the most vascular organs, containing billions of capillaries. The dense vasculature in brain ensures adequate nutrient and oxygen supply for proper neuronal function, and also protects the brain from unwanted and possibly toxic substances by creating a cellular barrier between the brain and the rest of the body, named as blood-brain barrier (BBB) (van Tellingen et al, 2015). Based on structure and function, the BBB comprises of two different barriers. First, a physical barrier is created by 'non-fenestrated' endothelial cells connected by 'tight junctions', which restrict the passive diffusional entry of polar solutes and macromolecules from blood to brain parenchyma (Abbott et al, 2010; Korfel & Thiel, 2007). A 'continuous' layer of these specialized endothelial cells is enclosed by basement membrane. Close interactions with the surrounding astrocyte endfeet, pericytes, and microglia are required for the induction and maintenance of BBB (Abbott et al, 2010; Korfel & Thiel, 2007). Second part of BBB is formed by the membrane transporters (ATP binding cassette (ABC) transporters) located in the luminal or abluminal membranes of the endothelial cells, which actively efflux some of the passively diffusing polar solutes either after or during their diffusion across the BBB (Abbott et al, 2010). According to one estimate approximately 60% of the commercial drugs are pumped out from the brain via ABCB1 transporter (P-glycoprotein or P-gp) (van Tellingen et al, 2015). Generally, the increased lipophilicity, and smaller molecular size correspond to better physical BBB penetrability of a drug, however, the high lipophilicity can also increase their efflux by ABC transporters (Abbott et al, 2010; Korfel & Thiel, 2007).

The normal BBB function can be altered under certain CNS pathologies including brain tumors. Abnormal blood vasculature, comprising of fenestrated or non-fenestrated endothelial cells that can be arranged in a continuous stretch or contain inter-endothelial gaps, are often found in place of normal BBB in the malignant brain tumors (referred to as blood-brain tumor barrier - BBTB) (van Tellingen et al, 2015). The tumor core in HGGs contains leaky BBTB, making them accessible to the contrast enhancing MRI agents and other macromolecules including certain drugs. However, the BBTB in LGGs and the invasive regions of the HGGs (outside the tumor core and into the normal brain tissue) often resemble the normal 'intact' BBB. Moreover, certain treatments such as surgery and anti-angiogenesis therapies can normalize the BBTB (van Tellingen et al, 2015). Thus, intact BBB and BBTB impose major hurdles in the sufficient delivery of potential therapeutic drugs to the brain tumor cells. Additionally, the tumor cells may abnormally express the drug efflux transporters to further aggravate the drug-resistance in gliomas (van Tellingen et al, 2015).

Several strategies have been under investigation to either bypass or transiently open the BBB so as to improve delivery of therapeutic agents

to the brain tumors. Traditionally, drugs have been delivered intracranially using catheters or needles, therefore bypassing the BBB (van Tellingen et al, 2015). However, these methods are highly invasive, require complicated surgeries, and are unsuitable for therapeutic agents that require repeated delivery (Timbie et al, 2015). The intra-nasal delivery is a completely non-invasive method to bypass BBB for delivery of drugs to the brain parenchyma (Timbie et al, 2015). However, its widespread use is limited by the inconsistent and inefficient delivery to particular regions in the brain, and low drug absorption ability of the nasal epithelium (Timbie et al, 2015). Among the non-invasive chemical methods that can breach the BBB, intra-arterial injection of mannitol (hyperosmotic agent) or vasodilators have been examined (van Tellingen et al, 2015). These agents open the BBB by shrinking the endothelial cells, allowing a window of up to 5 hours for delivery of therapeutic agents to the brain (van Tellingen et al, 2015). However, due to uncontrolled entry of solutes and large molecules, this form of BBB opening can lead to fluid accumulation in the brain, and thereby neurotoxicity (van Tellingen et al, 2015). Interestingly, focused delivery of low frequency ultrasound waves (FUS) through the skull has been used to transiently open the BBB in a specific (reasonably small) region of the brain, without affecting the neighboring brain tissue (Poon et al, 2016). This non-invasive method when combined with intravascular microbubbles (MBs) can significantly improve the usability of FUS, by reducing the required ultrasound acoustic energy and minimizing the skull heating side effects (Poon et al, 2016; Timbie et al, 2015). The microbubble guided FUS approach involves stretching of the blood vessels, which is caused by the expansion and contraction of MBs passing through the vessels in response to ultrasound (at the focused region) (Poon et al, 2016). Intra-operative magnetic resonance (MR) imaging can be used to locate malignant regions in the brain, target FUS to open BBB particularly in those regions, and allow concomitant drug delivery to the tumors (Poon et al, 2016). Since MBs are gas bubbles lined by lipid or protein on the outside, attempts have been made to encapsulate drugs (e.g. BCNU) within MBs that would allow targeted and enhanced drug delivery to the malignant regions (Timbie et al, 2015). Other methods to circumvent BBB include encapsulation of therapeutic agents, such as drugs, anti-sense RNA (siRNA), or DNA into nanoparticles (NPs) conjugated with BBB-penetrating ligands (Timbie et al, 2015). NP encapsulation reduces the toxic side effects of the therapeutic compounds, but if administered systemically, NPs usually require very high concentration to achieve efficient delivery to the brain (Timbie et al, 2015). Nevertheless, FUS with MBs is shown to enhance the delivery of 60 nm brain-penetrating nanoparticles in animal models (Nance et al, 2014). Thus, a combination of approaches that enhance BBB-penetration and reduce the toxic side effects of cytotoxic anti-cancer agents would broaden the range of possible treatment options for the fatal brain tumors.

2.3. Colorectal cancer (CRC)

The cancers that originate in the colon and rectum parts of the large intestine are collectively named as colorectal cancers (CRC). Polyps, which are benign noncancerous growth of glandular cells on the inner epithelial lining of colon and rectum, can frequently originate in individuals with old age and in those with associated family history (Risio, 2010). Those polyps that have the potential to progress to carcinoma are called adenomatous polyps or adenomas. It has been estimated that fewer than 10% of adenomas progress to carcinoma (Risio, 2010). CRC is a slow growing malignancy and it usually takes more than 10 to 20 years for adenomas to give rise to carcinoma (Winawer & Zauber, 2002). Somatic or genetic mutations in the *APC* (adenomatous polyposis coli) gene (>90%) or inactivation of APC/β-catenin pathway by other mechanisms occur in all CRCs. Activating mutations in kirsten rat sarcoma viral oncogene homolog (*KRAS)* (50%), inactivating mutations in *TP53* (50-70%) and allelic loss of chromosome 18q (80%) are additional 'drivers' of CRC (Brenner et al, 2014; Lech et al, 2016). High frequency of mutations in *BRAF* (8-13%) and *PIK3A* (10-20%) are also detected in CRC (Brenner et al, 2014).

According to WHO histological classification there are eight sub-types of colorectal carcinomas, with adenocarcinoma being the most frequently detected subtype (~96% cases) (Stewart et al, 2006). Globally, CRC is the second most diagnosed malignant cancer in females, and the third in males (Ferlay et al, 2015; Torre et al, 2015). Overall in 2012, nearly 1.3 million CRC cases were diagnosed, and 694000 deaths reported worldwide (Torre et al, 2015). The incidence and mortality rates for CRC are higher in men than in women, and the risk increases with age (Torre et al, 2015). Based on the TNM classification, the CRC tumors are grouped between stage I – IV at the time of diagnosis (Brenner et al, 2014; Compton, 2003; WHO, 2000). TNM stands for: T, the extent of primary tumor invasion into walls (tissue layers) of the intestine; N, the extent of tumor cell spread to the regional lymph nodes; and M, metastasis (tumor spread) to distant lymph nodes and organs (Compton, 2003; WHO, 2000).

The standard of care for CRC is surgery in more than 80% of cases (Compton, 2003). For high risk stage II and stage III CRC patients, adjuvant or neo-adjuvant treatments such as chemotherapy or radiotherapy are usually recommended, and they have beneficial effects on patient survival (Brenner et al, 2014). Chemotherapy compounds that have displayed improved CRC patient outcome are: 5-fluorouracil (5-FU), capecitabine, oxaliplatin and irinotecan (Brenner et al, 2014). Moreover, molecularly targeted therapies based on inhibition of VEGF (bevacizumab) or EGFR (cetuximab and panitumumab) in combination with chemotherapy compounds is suggested for patients with wild-type *KRAS* tumors (Arnold & Seufferlein, 2010; Brenner et al, 2014). Some of

these combination therapy regimens have shown improved survival in a sub-group of patients with characteristic tumor cell genetic status (Arnold & Seufferlein, 2010).

2.3.1. Colorectal cancer biomarkers

According to the National Cancer Institute (NCI), biomarker is defined as: "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease" (www.cancer.gov). Specific alterations in the tumor DNA, RNA or proteins are frequently tested for their potential use as a genetic, epigenetic or protein biomarker. A biomarker can be useful for screening patients for the presence of a disease at an early stage (screening/ diagnostic biomarker) (Lech et al, 2016). Alternatively, a biomarker can be helpful for patient prognosis, for instance to identify patients at higher risk of tumor recurrence (prognostic biomarker). Lastly, a biomarker can help to direct patients to certain arms of treatment modalities or to monitor the efficacy of a given treatment (predictive biomarker) (Italiano, 2011; Lech et al, 2016).

Currently there is no internationally accepted non-invasive diagnostic biomarker for CRC (detectable in blood or stool), though prognostic and/or predictive biomarkers have been recommended in some cases (Lech et al, 2016). The expression of a tumor-associated antigen, carcinoembryonic antigen (CEA) is highly elevated in CRC, some other cancers and inflammatory conditions (Laurence et al, 1972; Lech et al, 2016). CEA is the only marker that is currently recommended by many international cancer organizations (ASCO, ESMO and NCCN) as a prognostic marker indicative of more aggressive and recurrent CRC (Becerra et al, 2016; Lech et al, 2016). The CEA upregulation is usually not detectable in stage I CRC, therefore, its usability as prognostic biomarker is mostly applicable to the stage II and/or III CRCs (Becerra et al, 2016; Lech et al, 2016). Other glyco(protein) cancer antigens such as CA19-9, CA72-4, CA242 and CYFRA21-1 display much lower sensitivity and specificity as independent CRC biomarkers, however, a panel including CEA and these antigens provides better CRC diagnostic accuracy than any of these biomarkers alone (Lech et al, 2016).

Intense research effectors to understand the mechanism of CRC progression and therapy response have uncovered key molecular changes that have the potential to be used as prognostic or predictive biomarkers. Microsatellite instability (MSI), which is characterized by several insertion and deletion changes in the repeated DNA sequences (microsatellites), often arises due to inactivation of the cell's mismatch repair machinery (Brenner et al, 2014). Inactivating mutations or promoter hypermethylation of *MLH1* have been linked with the high level of MSI (MSI-H). The CRC patients with MSI-H have favorable prognosis

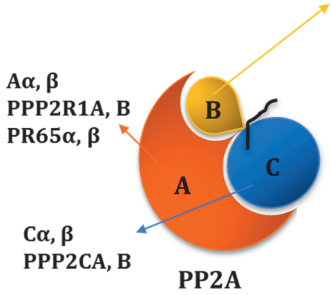
than those with stable microsatellites (Lech et al, 2016). In addition to the prognostic value, the presence of MSI-H also predicts resistance towards 5-FU and possible response to irinotecan based therapy (Brenner et al, 2014). Another tumor cell defect, chromosome 18q allelic loss or deletion associates with poor survival of stage III CRC patients (Sarli et al, 2004). The mutational analysis of *KRAS* gene (predictive biomarker) is nowadays a clinical routine for metastatic CRC patients. The patients with wild-type *KRAS* tumors are predicted to be better responders to panitumumab or other anti-EGFR therapy than those carrying oncogenic constitutively active K-Ras G12V mutation (K-Ras^{G12V}) (Arnold & Seufferlein, 2010). Similarly, a Ras downstream target, B-Raf^{V600E} activating mutation, as well as mutations in *PIK3A* gene predict non-responsiveness to anti-EGFR targeted therapies (Arnold & Seufferlein, 2010; Lech et al, 2016).

2.4. Protein phosphatase 2A (PP2A)

Protein phosphorylation is a post-translational modification event, which is reversibly carried out by the action of phosphorylating protein kinases (writers) and the phosphate-group remover protein phosphatases (erasers). Till date, over 500 protein kinases have been identified, out of which ~400 kinases carry out phosphorylation at serine or threonine residues (PSTKs) (Brautigan, 2013; Shi, 2009). The remaining kinases are mainly classified as tyrosine kinases (PTyKs), and a very small fraction as histidine and lysine kinases (Roskoski, 2015; Shi, 2009). There are ~140 known protein phosphatase catalytic subunits, and based on the substrate specificity they fall into four categories: 1) serine/threonine phosphatases (PSTPs), 2) tyrosine phosphatases (PTyPs), 3) dual specificity phosphatases (DUSPs), and 4) histidine phosphatases (Klumpp & Krieglstein, 2009; Shi, 2009). The total number of PST-phosphatases (~40) that oppose the Ser/Thr protein phosphorylation events carried out by a large number of PST-kinases (~400), appears to be small (Moorhead et al, 2007; Sents et al, 2013). However, phosphatases often function as multi-subunit complexes, and can be bound to one of the many regulatory subunits that direct the phosphatase complex to a limited number of target proteins. By virtue of numerous regulatory subunit partners, one phosphatase catalytic subunit can be part of tens or even hundreds of phosphatase holoenzyme complexes. Thus, the total number of specific multi-subunit phosphatase complexes would easily outnumber the kinases in a cell. Protein phosphatase 2A (PP2A) along with protein phosphatases PP1, PP2B (or calcineurin), PP2C, PP4, PP5 and PP6 comprise the major PSTP activity in a cell (Brautigan, 2013).

2.4.1. PP2A structure and function

Structurally, PP2A consists of three different subunits: a catalytic C-subunit (PP2A-C), a scaffolding A-subunit (PP2A-A or PR65), and a regulatory B-subunit. There are two isoforms, α and β, for the A and C subunits each, which are encoded by genes located on different chromosomes (Figure-2). The expression levels of these isoforms differ depending on the cell and tissue context, though Aα and Cα are more abundant than their Aβ and Cβ counterparts (Eichhorn et al, 2009; Sangodkar et al, 2015). There are 87% and 97% sequence similarities between the two isoforms of A and C subunits respectively (Eichhorn et al, 2009). The Aβ isoform expression is higher during the early stages of vertebrate development than in the adult tissues. Interestingly, some of the functions carried out by Aβ are unique, and cannot be compensated for by Aα (Eichhorn et al, 2009). The B-subunits have been broadly categorized into four families, each containing about 3-5 different isoforms and additional splice variants, altogether generating at least 26 different B-subunits (Eichhorn et al, 2009). A number of nomenclature systems have been in use for the PP2A subunits specifically for the B-subunits, which are illustrated in Figure-2. The following B-subunit family names will be used in this thesis hereafter: 1) R2/PR55, 2) R5/PR61, 3) R3/PR72 and 4) Striatin (STRN). Based on the number of known A, C and B subunits, PP2A can theoretically exist as nearly 100 different holoenzyme complexes.



B	R2A R2B R2C R2D	PPP2R2A PPP2R2B PPP2R2C PPP2R2D	PR55α PR55β PR55γ PR55δ	B55α B55β B55γ B55δ
B'	R5A R5B R5C R5D R5E	PPP2R5A PPP2R5B PPP2R5C PPP2R5D PPP2R5E	PR61α PR61β PR61γ1/2 PR61δ PR61ε	B56α B56β B56γ1/2 B56δ B56ε
B''	R3A R3B R3C	PPP2R3A PPP2R3B PPP2R3C	PR130/72 PR70/48 G5PR	
B'''	Striatins	STRN STRN3 STRN4	PR110 PR93	Striatin Striatin-3 or SG2NA Striatin-4

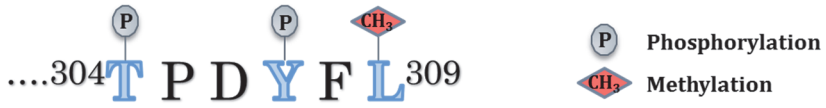


Figure-2. Structural components of a PP2A complex. *The subunit names derived from various nomenclature systems are summarized. The bottom picture illustrates the possible post-translational modifications of PP2A-C subunit carboxy-terminal tail (6 residues).*

The major function of PP2A-A subunit is to provide a scaffold or structural support for the binding of C and B subunits. For this function, PP2A-A contains 15 tandem HEAT repeats of ~40 amino acid residues arranged into two anti-parallel hydrophobic α-helices, which provide a characteristic horse-shoe shape to this subunit (Sangodkar et al, 2015). The PP2A-C subunit contains the catalytic phosphatase activity, and for the hydrolysis of Ser/Thr phosphate esters, it requires the presence of two manganese (Mn^{2+}) ions in its active site (Xing et al, 2006). PP2A-C binds to the HEAT repeats 11-15 of the PP2A-A subunit (located at one end of the horse-shoe) in a manner that the active site of PP2A-C is in close proximity to other end of the horse-shoe, where B-subunit binds to the PP2A-A subunit (Cho & Xu, 2007; Xing et al, 2006). The B-subunits contain putative substrate binding sites, and function as a regulatory partner whose binding directs the PP2A activity to a distinct set of substrates (i.e. regulators of substrate specificity) (Slupe et al, 2011). The B-subunits from different families have distinct structures and different binding sites on the PP2A-A subunit (Sangodkar et al, 2015). In a holoenzyme, B-subunits can also make contact with the PP2A-C subunit, the extent and mode of which differs among the B-subunit families. However, the proximity of B and C subunits in a holoenzyme (as explained earlier) illustrates their role in defining substrate specificity (Saraf et al, 2010; Slupe et al, 2011; Xu et al, 2006). B-subunit expression differs greatly based on the cell, tissue and developmental context (Schmidt et al, 2002; Zwaenepoel et al, 2008). Therefore, PP2A can modulate different signaling pathways and cellular functions dependent on the cellular context.

The major catalytic activity of PP2A is to carry out dephosphorylation on serine and threonine residues (PSTP). However, a transient tyrosine phosphatase activity (PTyP) has also been detected under certain physiological conditions (Guo & Damuni, 1993). The PSTP activity of PP2A is directed against different phosphoprotein targets, whereas, the PTyP activity is implicated in autodephosphorylation of PP2A at Tyr307 (Figure-2) (Chen et al, 1992) (mentioned in more detail later in the review).

PP2A is required for the proper functioning of a number of signaling pathways involved in cell division, cell cycle regulation, DNA damage response, stress response (for example hypoxia), growth factor response, cell adhesion and survival as well as programmed cell death including apoptosis and autophagy (Eichhorn et al, 2009; Janssens & Goris, 2001; Junttila et al, 2008; Klumpp & Krieglstein, 2002; Kurimchak & Grana, 2015; Nikolova-Karakashian & Rozenova, 2010; Sents et al, 2013). PP2A is also essential for neuronal signaling and development of brain, and its dysfunction has been associated with the CNS disorders (Sontag & Sontag, 2014). It is noteworthy that these distinct functions are carried

out by specific PP2A complexes being directed by the B-subunits. For example, R2A/PR55α and some R5/PR61 family members are involved in PP2A function in the brain (Slupe et al, 2011). The tissue-specific differential expression of B-subunits provides an additional layer of 'specific PP2A function' in a cell (Eichhorn et al, 2009; Janssens & Goris, 2001; Sents et al, 2013; Virshup & Shenolikar, 2009).

2.4.2. PP2A biogenesis and regulation of substrate specificity

The investigations into the mechanism of PP2A biogenesis and regulation of its catalytic activity have recognized an ever-increasing complexity, with the identification of new mechanisms and the discovery of new functions associated with the already existing regulatory factors. Overall, PP2A is regulated by various post-translational modifications (PTMs) and protein-protein interactions, which affect the stability and activity of this enzyme complex. The PTMs of PP2A-C subunit are mainly concentrated on its carboxy-terminal tail. These include a very unique methylation at carboxyl group of the terminal Leu309, and phosphorylation at Thr304 and Tyr307 (Figure-2, lower panel). Major PP2A associating proteins (also called as PP2A modulators or non-canonical regulatory subunits) involved in the holoenzyme biogenesis are: alpha4 (α4) (also called IGBP1 or Tap42), target of rapamycin signaling pathway regulator-like 1 (TIPRL1), leucine carboxyl methyltransferase-1 (LCMT-1), protein phosphatase methylesterase-1 (PME-1), and protein phosphatase 2A activator (PTPA) (Sents et al, 2013).

The heterotrimeric form of PP2A (ACB subunit complex), also called as holoenzyme, is the most abundant form of PP2A in mammalian cells (Kremmer et al, 1997; Murata et al, 1997; Yoo et al, 2007). The generation of an active PP2A complex proceeds through extensive regulatory steps. A simplified view is presented in Figure-3.

The PP2A-C subunit (monomeric or free) is synthesized in an inactive and possibly unstable form (Fellner et al, 2003; Murata et al, 1997; Sents et al, 2013). The unstable free PP2A-C subunit is stabilized by binding with protein α4, which protects PP2A-C from Mid1 (E3-ubiquitin ligase)-mediated polyubiquitination, and subsequent degradation (McConnell et al, 2010; Trockenbacher et al, 2001). Interestingly, the PP2A-C subunit bound to α4 can be catalytically active and dephosphorylate specific substrates such as mitogen-activated protein kinase kinase 3 (MEK3), Mid1 and possibly others (Sents et al, 2013). Binding with PP2A-A subunit can also stabilize the free PP2A-C, generating a core dimer (AC) complex (Figure-3-1.). Alternatively, the AC dimer can be formed after dissociation of the PP2A-C from α4 (Figure-3-1.).

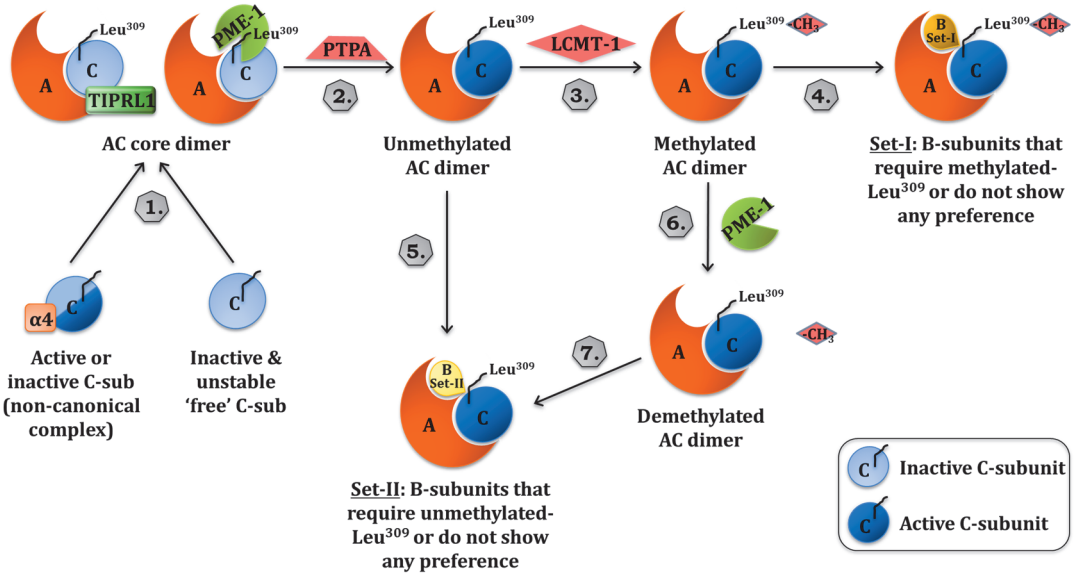


Figure-3. A model of PP2A biogenesis, and the function of various PP2A modulatory proteins in this process.

The AC dimer is under the inhibitory regulation of proteins such as TIPRL1 and PME-1, which (at least partially) prevent the promiscuous catalytic activity of PP2A-C before it is bound to a substrate recognizing B-subunit (Hombauer et al, 2007; Sents et al, 2013). Just like α4, TIPRL1 (also called TIP or Tip41) can associate with the PP2A-C, but in AC dimer form (Sents et al, 2013; Smetana & Zanchin, 2007). Atypical complexes of TIPRL1-PP2A-α4 have also been found to exist in a cell. However, the TIPRL1 binding is mainly considered inhibitory to the PP2A catalytic activity. Similarly, PME-1 can also stabilize PP2A-AC dimers and retain them in catalytically inactive form (Longin et al, 2004; Sents et al, 2013). The PME-1 or TIPRL1 bound PP2A-AC dimers are activated by the action of another enzyme, PTPA (Figure-3.2). Interestingly, PP2A-C within a core dimer requires a conformational change to generate an active conformation (Longin et al, 2004; Stanevich et al, 2011). This activation is carried out by a peptidyl-prolyl cis/trans-isomerase PTPA (Hombauer et al, 2007; Jordens et al, 2006; Leulliot et al, 2006; Longin et al, 2004). Several other mechanisms have been proposed by which PTPA can activate PP2A (described later in section 2.4.3.2.1).

The PP2A-AC dimer activated by the action of PTPA is ready for binding of a B-subunit. However, the choice of B-subunit binding depends on the amino acid modifications (PTMs) on the PP2A-C subunit carboxy-terminal tail. Importantly, the methylation of PP2A-C Leu309 plays a very crucial role in the PP2A biogenesis as well as regulation of substrate

specificity (Figure-3 and 4). A methyltransferase enzyme LCMT-1 adds methyl group (methylation) to the free carboxyl group of PP2A-C Leu309 (De Baere et al, 1999), whereas in a reversible reaction another enzyme PME-1 removes this methyl group (demethylation) (Ogris et al, 1999) (Figure-4.1). The function of this modification is described in detail later in this review (section 2.4.3.2). Briefly, binding of some B-subunits to the core dimer requires the presence of a methylated-Leu309 (Janssens et al, 2008). Conversely, some B-subunits prefer the demethylated-Leu309, and some others do not show any preference with regard to the Leu309 methylation status (Janssens et al, 2008). Thus, the PP2A-C methylation status affects the preferential recruitment of B-subunits to generate distinct active PP2A holoenzyme complexes. This implies that if PTPA-reactivated PP2A-AC dimer complexes are methylated by LCMT-1 (Figure-3-3.), they would generate trimeric PP2A complexes containing a distinct set of B-subunits (set-I: those favoring methylated PP2A-C or without any preference) (Figure-3-4.). On the other hand, the PTPA-activated PP2A-AC dimers which do not get methylated by LCMT-1 (i.e. unmethylated AC dimers), would bind a different set of B-subunits (set-II: those favoring unmethylated PP2A-C or without any preference) (Figure-3-5.) (Janssens et al, 2008). Alternatively, PME-1 can bind to the methylated PP2A-AC dimers and demethylate PP2A-C Leu309 (Figure-3-6.) (Longin et al, 2004; Tolstykh et al, 2000; Yu et al, 2001). These demethylated PP2A-AC dimers would also preferably associate with the set-II B-subunits (Figure-3-7.). Importantly, different PP2A(B-subunit) complexes dephosphorylate different set of target proteins. Thus, the altered expression of LCMT-1 and PME-1 can dynamically shift the balance of distinct PP2A complexes in a cell, and affect the substrate specificity.

Notably, the PP2A holoenzyme has gone through several steps of regulation, and is highly specific for certain target proteins. However, the holoenzyme activity and substrate specificity can still be regulated by distinct mechanisms operational under certain physiological and subcellular conditions. Additionally, a number of PP2A inhibitory proteins have been characterized in human malignancies such as CIP2A, SET, ANP32A, ARPP19, and ENSA. These will be briefly discussed later in this review.

Apart from the Leu309 methylation, and B-subunit directed substrate specificity, PP2A function is affected by other post-translational modifications concentrated at the carboxyl-terminal 6 amino acid (^{304}TPDYFL^{309}) region of the PP2A-C subunit (Figure-2) (Janssens et al, 2008). The phosphorylation of PP2A-C at Thr304 and Tyr307 residues possibly inhibit formation of PP2A complexes containing R2/PR55 and R5/PR61 B-subunits respectively (Chen et al, 1992; Janssens et al, 2008; Sents et al, 2013). Conversely, PP2A-C mutational analysis

suggests that Tyr307 phosphorylation may enhance the recruitment of striatin family B-subunits (Yu et al, 2001). Very little is known about the kinases responsible for phosphorylation of Thr304, although CDK1 has been suggested to phosphorylate this residue during mitosis (Schmitz et al, 2010). Increased Tyr307 phosphorylation has been associated with the active Src and glycogen synthase kinase-3β (GSK-3β) kinases, and EGFR and insulin receptor signaling (Janssens & Goris, 2001; Xiong et al, 2013; Yao et al, 2012). The autodephosphorylation activity of PP2A has been suggested to act on these residues, and activate itself (Chen et al, 1992; Guo & Damuni, 1993). Interestingly, the absence of Tyr307 phosphorylation seems to be important but not absolutely necessary for methylation at Leu309 (Yu et al, 2001). A peroxynitrile dependent tyrosine nitration has been reported to inhibit tyrosine phosphorylation of PP2A-C and promote the phosphatase activity in endothelial cells (Wu & Wilson, 2009). It can be speculated that the tyrosine residues involved in this case could be Tyr307. The post-translational modifications are not only restricted to PP2A-C subunit. In one study performed in neuronal cells, phosphorylation of R2B/PR55β on three specific serine residues constrains this B-subunit in the cytoplasm (Merrill et al, 2013). However, the autodephosphorylation of these residues by PP2A results in translocation of the PP2A(R2B/PR55β) complexes into outer mitochondrial membrane (Merrill et al, 2013). This translocation results in mitochondrial fragmentation and sensitization of neuronal cells to apoptosis in response to additional neuronal insults (Merrill et al, 2013).

The methylation of PP2A-C Leu309 has been very recently suggested to promote PP2A-C ubiquitination and degradation in PME-1 knockout mouse embryonic fibroblast cells (Yabe et al, 2015). The demethylated PP2A-C is therefore speculated to promote association with α4 in order to stabilize the free PP2A-C subunit pool in the cell, as previously observed in yeast (Wu et al, 2000). Thus, there seems be a crosstalk between different PP2A regulatory mechanisms. Notably, in addition to PP2A, both α4 and TIPRL1 have been found to interact with and modulate the activity of PP4 and PP6 phosphatases (Nanahoshi et al, 1999; Rosales et al, 2015), and PME-1 has been found to interact with PP4 (Wandzioch et al, 2014).

2.4.3. Protein phosphatase methylesterase-1 (PME-1)

2.4.3.1. PME-1 structure and function

N-terminal protein methylations occurring on the amino-terminus (N-terminus) or side chain N-atoms of lysine or arginine residues of histone and some non-histone proteins are largely known (Bedford, 2006;

Clarke, 2013). A second group of methylation occurs on the carboxyl-group of (1) glutamate side chains, (2) modified arginine and cysteine residues (e.g. on Ras and Rho-GTPase proteins) and (3) on the C-terminal leucine residue of some protein phosphatase catalytic subunits, such as PP2A-C (Bedford, 2006; Clarke, 2013; Rounds et al, 2005). Protein carboxymethylation is a reversible event and is involved in the protein-protein interaction, regulation and signal transduction analogous to protein phosphorylation. The carboxymethylation (hereafter referred to as methylation) of PP2A-C at carboxyl group of C-terminal leucine 309 (Leu309) residue is carried out by a leucine specific methyltransferase LCMT-1 utilizing S-adenosylmethionine (De Baere et al, 1999), and is reversibly removed by a methylesterase PME-1 (Figure-4-1.) (Lee et al, 1996; Ogris et al, 1999).

PME-1 was the first eukaryotic carboxylmethylesterase to be characterized. It was purified from bovine brain extracts (Lee et al, 1996). This report was soon followed by the protein and cDNA sequencing of this methylesterase, and it was named as protein phosphatase methylesterase-1 (PME-1) (Ogris et al, 1999). PME-1 is a 44-KDa (386 residue) intracellular protein, which belongs to the subfamily of serine hydrolases containing a catalytic triad of Ser-Asp-His with Ser (Ser156 in PME-1) as an active site residue (Ogris et al, 1999; Xing et al, 2008). The PME-1 protein encoded by *PPME1* gene is conserved among eukaryotes, and its highest transcript expression is reported in brain and testis tissue extracts from mouse (Ogris et al, 1999). PME-1 contains an active nuclear localization signal (^{270}KRKK^{273}), which directs its predominant localization to the nucleus, where most of the demethylated PP2A pool has been detected in HeLa cells (Longin et al, 2008).

PME-1 methylesterase activity is insensitive to serine esterase inhibitors PMSF or DFP in cell-free assays (Lee et al, 1996; Ogris et al, 1999). The reason behind the esterase inhibitor insensitivity of PME-1, even though it is a serine esterase, was explained when the crystal structure of PME-1 was solved by Xing et al. In a free state, not bound to PP2A, PME-1 exists in an inactive conformation unaffected by esterase inhibitors (Xing et al, 2008). The binding of PME-1 to PP2A-C induces rearrangement of catalytic triad residues (Ser156, Asp181, and His349) into an active form. These structural rearrangements in PME-1 open the active site pocket so that the PP2A-C C-terminal ^{304}TPDYFL^{309} tail fits into this pocket in an appropriate orientation and in close proximity to the PME-1 catalytic residues (Xing et al, 2008).

The attempts to generate PME-1 knockout (KO) mouse have revealed that even though the knockout mice develop normally *in utero*, they cannot survive after birth (perinatal lethal) (Ortega-Gutierrez et al, 2008). This study suggested that PME-1 is the sole PP2A methylesterase

and that the demethylated PP2A is essential for normal cellular functioning. PME-1 KO mouse brain tissues also displayed differential phosphorylation of several proteins, especially those involved in signal transduction, transcriptional regulation and cytoskeleton (Ortega-Gutierrez et al, 2008).

2.4.3.2. Various modes of PP2A regulation by PME-1

The crystallographic studies have shown that PME-1 binds PP2A-C (from AC dimer) in its active site forming an interaction interface containing several hydrogen bonds and other covalent interactions (Xing et al, 2008). A point mutation of PME-1 at Arg369 to aspartic acid (R369D) results in complete loss of PP2A-C binding (Pokharel et al, 2015). PME-1 binding evicts manganese (Mn^{2+}) ions from the PP2A active site resulting in PP2A inhibition (Figure-4-3.) (Longin et al, 2004; Xing et al, 2008). The activation of PME-1 methylesterase activity requires its binding with PP2A-C (as explained earlier). Therefore, PME-1 binding to PP2A appears to be necessary for its methylesterase activity. Since okadaic acid (OA) and microcystin-LR also bind PP2A-C in the active site, their binding must compete with the PME-1 binding to PP2A-C (Xing et al, 2008; Xing et al, 2006). This competitive binding explains the OA and microcystin-LR mediated inhibition of PP2A-C demethylation by PME-1 in cell-free assays reported earlier (Lee et al, 1996; Ogris et al, 1999; Xing et al, 2008).

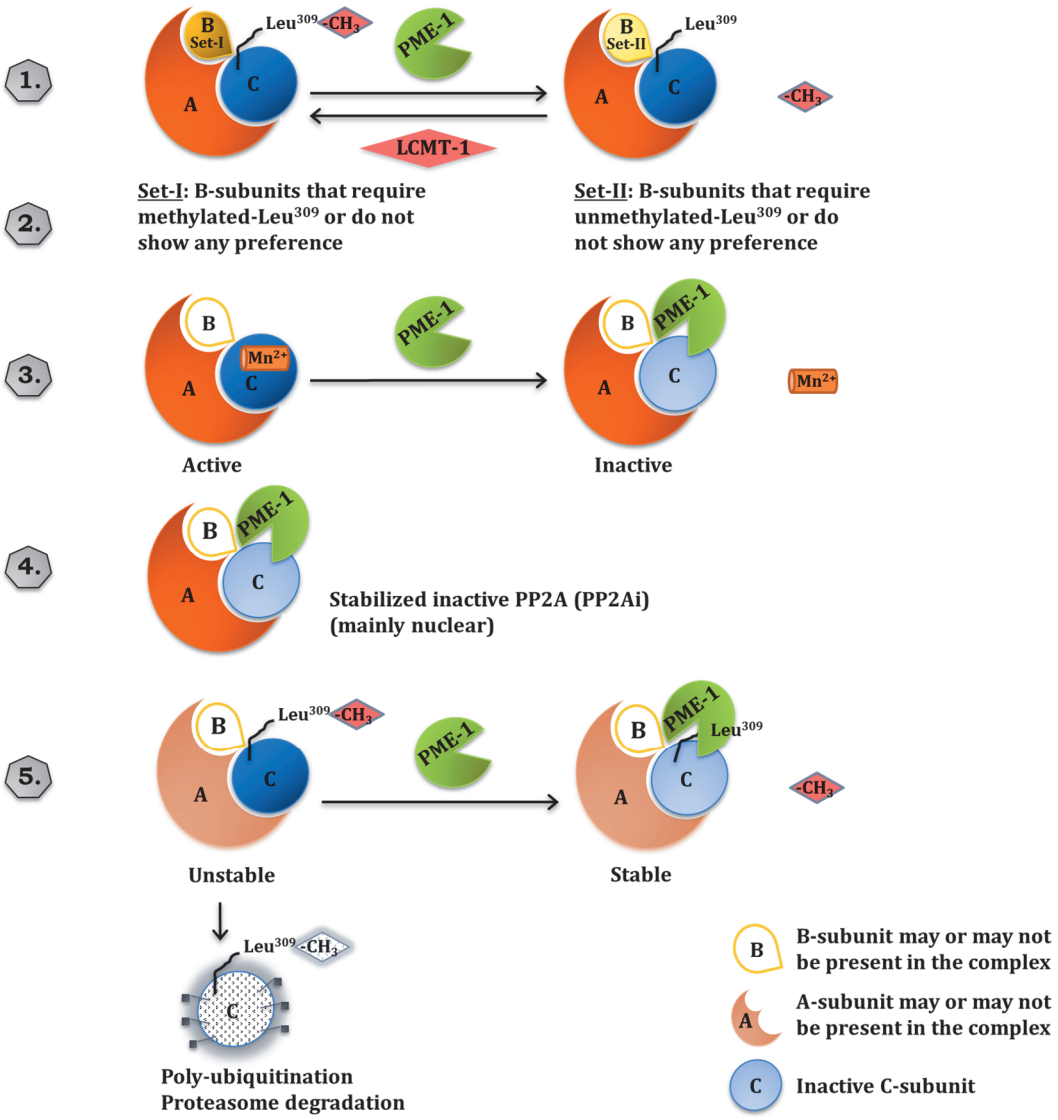


Figure-4. Various modes of PP2A regulation by PME-1. *(1.) PME-1 and LCMT-1 reversibly regulate PP2A-C Leu309 methylation (-CH3), and (2.) B-subunit selection, thereby influenc the substrate specificity. (3.) PME-1 binding removes Mn2+ ions from the PP2A active site and inhibit its activity. (4.) PME-1 binding maintains a stable and inactive PP2A pool in the nucleus. (5.) PME-1 protects PP2A-C from degradation. PME-1 functions presented here are linked with its functions in PP2A biogenesis.*

Earliest report suggested that the PP2A-C Leu309 methylation enhances the phosphatase activity of PP2A *in vitro* (Favre et al, 1994), so that the PME-1 mediated demethylation would lead to reduced PP2A activity. Several later reports found that the methylation of PP2A-C

Leu309 does not affect the phosphatase activity of PP2A towards several different substrates (De Baere et al, 1999; Ikehara et al, 2007; Tolstykh et al, 2000). However, the structural analysis of PME-1-PP2A complex, and the phosphatase activity assays performed at longer time points have confirmed that PME-1 inhibits phosphatase activity of PP2A towards phosphorylase a (Xing et al, 2008). It has been suggested that the PME-1 binding-mediated removal of Mn^{2+} ions from the active site of PP2A requires longer incubation time. In addition to this *in vitro* evidence, PME-1 silencing reduces PP2A-C demethylation as well as increases phosphatase activity of PP2A in RL95-2 cells (Wandzioch et al, 2014), and the MEK-associated PP2A complexes in HeLa cells (Puustinen et al, 2009). Additionally, a catalytically inactive mutant form of PME-1 (Ser156 to Ala) can bind methylated yet inactive PP2A (more strongly than the active wild-type PME-1), indicating that the PME-1-binding mediated PP2A inhibition function is independent of its methylesterase activity (Longin et al, 2008; Wandzioch et al, 2014). It also suggests that PME-1 dissociates from PP2A once the demethylation reaction is complete. Nevertheless, whether PME-1 mediated PP2A activity inhibition is cell type dependent has not been addressed.

Furthermore, the methylation status of PP2A-C affects the preferential binding of some regulatory B-subunits to the PP2A core dimer (Janssens et al, 2008; Mumby, 2001). The B-subunits belonging to different families display differential binding to PP2A core dimer depending on the Leu309 methylation status (Figure-6 2.). Initial experiments using PP2A-C Leu309 mutants and purified PP2A complexes have suggested methylation to be required for the binding of R5/PR61 and to a great extent for R2/PR55 subunits (Bryant et al, 1999; Tolstykh et al, 2000). Further studies have shown a less stringent requirement for methylated PP2A-C for the binding of R5/PR61 and R3/PR72 subunits, under the conditions where R2/PR55 exclusively binds methylated PP2A-C (Cho & Xu, 2007; Longin et al, 2007; Xing et al, 2006; Xu et al, 2006; Yu et al, 2001). The striatin family subunits, such as Striatin (STRN) and SG2NA, presumably do not require methylated PP2A-C to generate heterotrimeric complexes (Yu et al, 2001). Strikingly, some indicative data shows that striatin family subunits might even prefer the unmethylated PP2A-C (Yu et al, 2001). Several contradictory *in vitro* and *in vivo* studies have been reported over the years confirming or disproving the above mentioned view (Bryant et al, 1999; Cho & Xu, 2007; De Baere et al, 1999; Ikehara et al, 2007; Longin et al, 2004; Tolstykh et al, 2000; Wu et al, 2000; Xing et al, 2006; Xu et al, 2006; Yu et al, 2001). A great diversity of the PP2A B-subunit isoforms, their cell and tissue dependent expression pattern, as well as the complexities of PP2A regulatory mechanisms have contributed to the lack of systematic studies in the past. The next generation technologies might provide

essential tools to characterize the mechanistic details of PME-1 regulated PP2A function.

The complex relationship between PME-1 and PP2A is further strengthened by some paradoxical observations. In yeast, *PPE1* (human PME-1 homolog) deletion increases PPH21 (human PP2A homolog) methylation, but reduces its phosphatase activity (Hombauer et al, 2007). The PME-1 KO mouse brain and peripheral tissues are virtually completely depleted of demethylated PP2A. However, depending on the phosphorylated substrate used, the phosphatase activity of PP2A isolated from the PME-1 KO mouse brain tissues has been either lower or similar to the wild type mouse (Ortega-Gutierrez et al, 2008). Similarly, downregulation of PME-1 in skeletal muscle cells renders the PP2A complexes incompetent in dephosphorylating the target Na^+,K^+-ATPase (NK) α-subunit (Benziane et al, 2012). These observations indicate that the loss of PME-1 might affect the composition of active PP2A complexes, resulting in reduced PP2A activity towards certain phospho-substrates and enhanced activity towards some others. Therefore, the difference in the type of phospho-substrate used and the cellular (*in vivo*) or chemical (*in vitro*) background might affect the outcome of phosphatase activity assays. Consistent with this view, the PME-1 KO mouse embryonic fibroblasts (MEFs) showed increased association of R2A/PR55α with PP2A-A and PP2A-C subunits as compared to wild-type MEFs, but no difference was observed in the R5A/PR61α binding (Yabe et al, 2015). Additionally, even though the overall PP2A activity towards artificial phospho-substrates is not affected in the PME-1 KO MEFs, the EGF-stimulated ERK and AKT phosphorylation (i.e. phosphatase activity towards these specific proteins) is strongly inhibited in these cells (Yabe et al, 2015), and as previously reported in cancer cells (Puustinen et al, 2009; Wandzioch et al, 2014).

It has been recently reported that PME-1 methylesterase activity also protects PP2A-C from ubiquitin/proteasome degradation in MEFs and certain type of cancer cells (Figure-4-5.) (Yabe et al, 2015). The PME-1 KO MEFs as well as the heart and liver tissue from P0 mice show reduction in total PP2A-C levels (Ortega-Gutierrez et al, 2008; Yabe et al, 2015). These findings provide another explanation why PME-1 inhibition or downregulation displays reduced PP2A phosphatase activity in some studies.

2.4.3.2.1. Interplay of proteins regulating PP2A methylation and activity

The PME-1 mediated regulation of PP2A is highly influenced by at least two other endogenous proteins, namely LCMT-1 (De Baere et al, 1999) and PTPA (Jordens et al, 2006; Longin et al, 2004; Sents et al, 2013). It

has been demonstrated that under certain circumstances, a pool of PP2A complexes, containing core AC-dimers and possibly ACB-heterotrimers, exists in an inactive conformation (PP2Ai) stabilized by PME-1 binding (Figure-4-4.)(Hombauer et al, 2007; Longin et al, 2004; Longin et al, 2008; Ogris et al, 1999; Tolstykh et al, 2000). Since majority of PME-1 expression is found in the nucleus, it is suggested that the PP2Ai pool mainly exists in the nucleus. As described earlier, the main function of LCMT-1 is to catalyze the methylation at carboxyl-terminal Leu309 residue of PP2A-C, and direct the B-subunit recruitment to generate active PP2A complexes (De Baere et al, 1999). However, it has been demonstrated that before this reaction could begin, PP2A-C within a core dimer is activated by conformational change carried out by a peptidyl-prolyl cis/trans-isomerase PTPA (Figure-3) (Longin et al, 2004; Stanevich et al, 2011). PTPA is also called as phosphotyrosyl phosphatase activator of PP2A or phosphatase two A phosphatase activator (Hombauer et al, 2007; Jordens et al, 2006; Leulliot et al, 2006; Longin et al, 2004). In addition to the prolyl-isomerase activity, PTPA can activate the intrinsic phosphotyrosyl phosphatase (PTyP) activity of PP2A leading to dephosphorylation of PP2A-C Tyr307 (or possibly by PTP1B), which is another important player in determining B-subunit binding and substrate recognition by active PP2A (Fellner et al, 2003; Luo et al, 2013; Van Hoof et al, 1994). PTPA can reactivate the Ser/Thr phosphatase (PSTP) activity of PP2Ai pool in the presence of ATP and Mg^{2+} by displacing PME-1 (Longin et al, 2004). Therefore, upon activation PP2Ai can be made available for dephosphorylation of some phosphoprotein targets, under certain physiological conditions. Notably, the PP2Ai pool activated by PTPA/ATP- Mg^{2+} (Figure-4-4.) is possibly distinct from the other PME-1-bound inactive PP2A pool (Figure-4-3.) that can be activated by Mn^{2+} (Longin et al, 2004). PTPA binding to PP2A core dimer has been proposed to induce a composite ATPase activity which might be required for the PTyP and PSTP activities of PP2A (Chao et al, 2006; Guo et al, 2014; Van Hoof et al, 1994).

Taken together, PME-1 stabilizes inactive pool of PP2A-C and participates in PP2A biogenesis (Figure-3 and 4). PME-1 inhibits PP2A activity by direct binding, and affects its substrate specificity by regulating the PP2A-C Leu309 methylation and the assembly of specific B-subunit-containing trimeric complexes. PP2A stabilization by PME-1 might involve protection of PP2A-C from degradation. Interestingly, various PP2A regulatory proteins associated with its methylation including PME-1 have been isolated from animal brain where their highest expression has been reported, reflecting the importance of this signaling event in the normal cellular functions in the brain and their possible implications in brain disorders such as Alzheimer's disease and gliomas.

2.4.3.3. Regulation of PME-1

Two independent studies have indicated that PME-1 can be regulated by phosphorylation, however, by different mechanisms. First, using back phosphorylation technique in OK (opossum kidney) cells, SIK1 (salt-inducible kinase 1) was shown to phosphorylate PME-1 (Sjostrom et al, 2007). This phosphorylation possibly results in dissociation of PME-1 from PP2A. The active PP2A in turn dephosphorylates NK α-subunit and activates the NK ATPase enzyme (involved in the regulation of cell volume) (Sjostrom et al, 2007). Here, a calcium calmodulin-dependent kinase (CaMK) was suggested to phosphorylate and activate SIK1. On the contrary, a recent study in HEK-293T cells illustrates that CaMK-I is responsible for phosphorylation of PME-1 at Ser15 residue (Lee et al, 2014). A negative feedback regulation was reported in this study, wherein; CaMK-I phosphorylates SIK2 (another SIK family kinase) and PME-1. This phosphorylation leads to SIK2 degradation and possibly increased PME-1 recruitment to PP2A complexes (which remain inactive). On the other hand, SIK2 bound PP2A complexes are active, and can inhibit PME-1 directly by dephosphorylation at Ser15 and indirectly by dephosphorylation of PME-1-kinase CaMK-I (Thr177). Given that the putative consensus sequence found around the phosphosite Ser15 of PME-1 can be targeted by AMP-activated protein kinase (AMPK), CaMK-IV and SIK1 in addition to CaMK-I, more studies would uncover the role of these kinases in the PME-1 mediated regulation of PP2A activity. Hereby, it has been indicated that the PP2A activity towards NK α-subunit in skeletal muscles is indeed regulated by AMPK in a yet unidentified PME-1 dependent manner (Benziane et al, 2012).

An oncofetal protein IMP1 (IGF2BP1 or ZBP1), highly expressed in cancers including choriocarcinoma, binds to and stabilizes PME-1 mRNA possibly leading to increased PME-1 expression in these cancers (Hsieh et al, 2013). Recently, PI3K-AKT signaling target GSK-3β, which is the major tau-kinase involved in Alzheimer's disease, has also been implicated in the inhibition of PME-1 expression at mRNA and protein level, most likely via some indirect mechanism which needs further investigation (Wang et al, 2015). In HEK-293FT cells, GSK-3β mediated downregulation of PME-1 and activation of LCMT-1 was suggested to activate PP2A via reduction in Leu309 demethylation (Wang et al, 2015). In stark contrast with this report, previously GSK-3β activation has been shown to upregulate PME-1 and downregulate LCMT-1 expression, thereby enhancing the inhibitory demethylated PP2A-C in HEK-293 and N2a cells (Yao et al, 2012). Therefore, the role of GSK-3β in the regulation of PME-1 remains disputed.

2.4.3.4. PME-1 chemical inhibitors

Two different families of PME-1 methylesterase (serine hydrolase) activity inhibitors have been described: the aza-β-lactum inhibitor ABL127 (Bachovchin et al, 2011a), and the sulfonyl acrylonitrile inhibitor AMZ30 (or ML136) (Bachovchin et al, 2011b). Both inhibitors covalently bind to the active site Ser156 of PME-1 and irreversibly inactivate it. Since inhibitor and PME-1 binding produces an adduct, the concentration of inhibitor required for efficient PME-1 inhibition in cells would be largely dependent on the PME-1 expression level in those cells, and should be considered for testing these compounds in different cell lines.

The IC_{50} values of ABL127 and AMZ30 for selective PME-1 inhibition are 10 nM and 500 nM respectively (Bachovchin et al, 2011a; Bachovchin et al, 2011b). Treatment with these inhibitors displayed reduction of demethylated PP2A-C levels in MEFs, HEK-293T, MDA-MB-231 and HeLa cells (Bachovchin et al, 2011a; Bachovchin et al, 2010; Xia et al, 2015; Yabe et al, 2015). However, increase in methylated PP2A-C upon inhibitor treatment can be only observed in cells expressing exogenous PME-1 (Bachovchin et al, 2011a; Bachovchin et al, 2011b). Additionally, AMZ30 treatment in HeLa cells leads to abnormal shortening of metaphase spindles, mitotic arrest, and cell death in a significant fraction of cells (Xia et al, 2015). In this study, the PME-1 silencing mediated mitotic arrest and cell death was less as compared to AMZ30 treatment (Xia et al, 2015), suggesting for possible off-target effects of AMZ30. PME-1 inhibition by either ABL127 or AMZ30 treatment inhibits the growth and migration of endometrial cancer cells *in vitro* (Rice & Pusey, 2015), but a single-dose intra-tumor ABL127 treatment has failed to suppress tumor growth in xenograft mice (Pusey et al, 2016). Conversely, PME-1 knockdown by siRNA or shRNA inhibits cancer cell growth not only in *in vitro* but also in *in vivo* endometrial cancer xenograft models (Pusey et al, 2016; Wandzioch et al, 2014).

Systemically delivered PME-1 inhibitors have not yet been tested in any human cancer xenograft studies. However, acute intraperitoneal treatment with ABL127 (2 hours) to mice results in 35% reduction in demethylated PP2A-C in brain tissue (Bachovchin et al, 2011a). This indicates that ABL127 is BBB-penetrable, a pharmacological property that is highly desirable for compounds targeting brain tumors and other CNS disorders. Whether these inhibitors can block the PME-1 binding-mediated inhibition of PP2A (Xing et al, 2008), remains to be investigated.

2.4.3.5. PME-1 in cancer

The oncogenic role of PME-1 was first reported in human gliomas, and the MAPK/ERK signaling was identified as a PME-1-regulated PP2A target pathway (Puustinen et al, 2009). PME-1 silencing in cultured cells increases association and activity of PP2A bound to MEK protein, resulting in reduced phosphorylation of MEK (p-MEK) and downstream ERK, (p-ERK) proteins. Furthermore, the MAPK/ERK pathway inhibition by PP2A is regulated by PME-1 at a level downstream of Ras (and growth factor receptors) and upstream of Raf (Puustinen et al, 2009). PME-1 silencing reduces MAPK/ERK phosphorylation and inhibits cell proliferation in Ras^{G12V} transformed cells, but not in $B\text{-}Raf^{V600E}$ or MEK^{DD} transformed HEK cells (Puustinen et al, 2009). It has been suggested that the cancer cells where activation of ERK occurs by activating mutations in Raf or by alternative Ras-independent pathways, the PME-1 knockdown would have no functional effects (e.g. reduction of cell proliferation).

In endometrial cancer cells, PP2A inhibition by PME-1 promotes cell proliferation by activation of the pro-survival ERK and AKT signaling (Wandzioch et al, 2014). These researchers also reported the first instance of *in vivo* tumor growth promoting function of PME-1 using subcutaneous ECC-1 endometrial carcinoma cell xenograft mouse model. Similarly, PME-1 depletion inhibits proliferation and anchorage-independent growth of HeLa cells, partially via inhibition of MAPK/ERK pathway (Puustinen et al, 2009). No apoptosis induction was detected in HeLa cells under these conditions. In glioblastoma cell lines, U118MG and T98G, even though a similar reduction in p-ERK and its target p-Elk-1 was observed, the reduction in cell proliferation was relatively small. It was also indicated that the PME-1 inhibition-mediated downregulation of ERK-pathway activity is dependent on the presence of growth factors (serum in the culture media). Thus, a certain degree of cell-type specific differences have been observed upon PME-1 depletion in cancer cells.

Using immortalized human embryonic kidney cells weakly transformed by downregulation of R5C/PR61γ (HEK-TERASB56γ) as a model system, LCMT-1 and PME-1 have been identified as negative and positive regulators respectively of malignant cell proliferation and survival under anchorage-independent conditions (Jackson & Pallas, 2012). LCMT-1 knockdown or PME-1 overexpression in this model showed partially similar results including reduction in PP2A-C Leu309 methylation, and increase in phosphorylation of AKT-Thr308 (but not Ser473), p70-S6 kinase (p70-S6K or RPS6KB), and rpS6 proteins, and in the anchorage-independent colony growth. Interestingly, none of these alterations were detectable under anchorage-dependent culture conditions. The *PPME1* mRNA stabilizing oncofetal protein IMP1 promotes migration and

invasion of choriocarcinoma JAR cells at least partly by enhancing PME-1 expression and possibly affecting the downstream signaling (Hsieh et al, 2013). Thus, depending on the cellular context, PME-1 seems to affect different cancer cell properties by regulating PP2A-effector pathways.
In addition to PP2A-C, PME-1 has also been shown to bind to the catalytic subunit of PP4 (PPP4C) in co-immunoprecipitation studies (Wandzioch et al, 2014). In contrast to the tumor suppressive function of PP2A, PP4 promotes tumor growth, suggesting the possibility that PME-1 may inhibit tumor growth via inhibition of PP4 activity. However, the overexpression of PP4 in endometrial cancer cells did not alter the PME-1-inhibition mediated reduction in cell proliferation, proposing PME-1 as a potential therapy target for endometrioid adenocarcinoma.

2.4.3.5.1. PME-1 expression in patient tumor material

Primary astrocytic glioma is the first cancer type where the expression of PME-1 had been assessed by immunohistochemical (IHC) methods (Puustinen et al, 2009). About fifty percent of the glioma tumor samples are PME-1 positive. In line with the cell culture studies, PME-1 expression shows a strong correlation with the p-MEK, p-Elk-1 and cell proliferation index (Ki67) in the glioma patient tumor samples. A strong association has been found between PME-1 staining and tumor grade (grade II to IV), suggesting an oncogenic role for PME-1 with increasing malignancy of glioma tumors. The analysis of *PPME1* mRNA and protein expression in a small panel (n = 30) of type I endometrioid adenocarcinoma samples has demonstrated enhanced expression of PME-1 in tumor samples as compared to matched normal adjacent tissue (Wandzioch et al, 2014).

A study in Chinese gastric and lung cancer patient cohort has described the existence of a small subset (~3%) of patients with amplification of *PPME1* (Li et al, 2014). Functional studies performed using *PPME1* amplified versus non-amplified gastric and lung cancer cell lines have suggested PME-1 regulated PP2A activity and pro-survival functions to be the driving factors in cancer cells which are dependent on PME-1 expression (synonymous with the oncogene addiction phenotype of cancers) (Li et al, 2014). PME-1 knockdown results in decreased PP2A-C demethylation, reduced AKT (Ser473) and ERK (Thr202) phosphorylation in *PPME1* amplified cancer cells (Li et al, 2014). Similarly, the inhibition of cell viability and induction of apoptosis upon PME-1 knockdown has also been observed specifically in *PPME1* amplified cells (Li et al, 2014).

In the absence of any other published reports, the CNA and mutations of *PPME1* were analyzed in the online cancer database cBioPortal (www.cbioportal.org). A very high frequency of *PPME1* amplification (21%) was found in neuroendocrine prostate cancer tumors from a very

recent study (Beltran et al, 2016). High frequency of *PPME1* amplification was also detected in carcinomas of serous ovarian (8.7%), esophageal (8.2%), head and neck squamous cell (5%), bladder urothelial (4.7%), breast (4.5%), and lung (3.5%) from published or provisional TCGA datasets (www.cancergenome.nih.gov). The *PPME1* amplifications in GBM and CRC were found in less than 0.5% of cases reported by TCGA network (Brennan et al, 2013; TCGA, 2012). In one CRC genome sequencing dataset, two mutations in *PPME1* were detected in two cases (2.8%) (Seshagiri et al, 2012). Although most of the PME-1 mutations including L79P and E75D found in CRC were missense mutations, no recurrent mutations of PME-1 could be detected in different cancers (www.cbioportal.org).

2.4.4. PP2A in cancer

Given the inhibitory function of PP2A on several growth promoting signaling pathways, it can be expected that the malignant cells would benefit from PP2A inhibition. In 1988, researchers from Japan first showed that a carcinogenic shellfish toxin, okadaic acid (OA), inhibits PP2A and PP1 phosphatases (Bialojan & Takai, 1988; Suganuma et al, 1988). This discovery was soon followed by a series of reports demonstrating PP2A as one of the major targets for DNA tumor virus proteins, such as SV40-ST, polyomavirus small and middle tumor antigens, and adenovirus E4orf4 (Pallas et al, 1990; Shtrichman et al, 1999; Walter et al, 1990). These viral proteins act by displacing specific B-subunits from the holoenzyme, therefore altering the PP2A activity towards specific substrates (Eichhorn et al, 2009; Westermarck & Hahn, 2008). A systematic analysis revealed that among the various PP2A(B-subunit) complexes, specific inhibition of R5A/PR61α, R5C/PR61γ and R3A/PR72/PR130 subunits contributes to the PP2A-inhibition mediated tumorigenesis by activating Myc, WNT and PI3K/AKT pathways (Sablina et al, 2010). It was an intriguing finding that different B-subunit containing PP2A trimers are targeted by different viral proteins, resulting in activation of distinct cellular pathways (Eichhorn et al, 2009; Rodriguez-Viciana et al, 2006; Westermarck & Hahn, 2008). Moreover, inhibition of PTPA results in disruption of PP2A holoenzyme biogenesis, which reproduced the PP2A inhibition in these transformation models (Sablina et al, 2010). Most recently, alteration in the PP2A methylation by knockdown of LCMT-1 or overexpression of PME-1 has been shown to contribute to tumorigenesis in human cells by activating AKT and p70/p85-S6K pathways (Jackson & Pallas, 2012).

Furthermore, cancer-specific inactivating mutations and deletions are known to occur in PP2A-A subunit genes (*PPP2R1A* and *PPP2R1B*) and to some extent in B-subunit genes, which establish PP2A as a classical tumor suppressor (Chen et al, 2005; Kalla et al, 2007; McConechy et al,

2011; Sallman et al, 2014; Sangodkar et al, 2015; Shih Ie & Wang, 2011; Shouse et al, 2010; Smits et al, 1992; Wang et al, 1998). Approximately forty percent of human endometrial tumors of high-grade serous type have been reported to display mutations in *PPP2R1A* (Aα) gene (McConechy et al, 2011). A relatively lower frequency of *PPP2R1A* somatic mutations (~5-10%) are detected in different histological types of ovarian cancers (McConechy et al, 2011; Rahman et al, 2013), breast and lung cancer, and melanoma (Calin et al, 2000). Genetic alterations in *PPP2R1B* (Aβ) gene are observed in ~15% of primary lung and colon cancers (Takagi et al, 2000; Wang et al, 1998), and less than 10% in breast cancer (Calin et al, 2000). The mutations or deletions in PP2A-A subunit are usually associated with reduced binding to PP2A-C subunit or in some cases to certain B-subunits (Ruediger et al, 2001a; b; Wang et al, 1998). Interestingly, in B-cell chronic lymphocytic leukemia (B-CLL) alternative splicing (by exon-skipping) has been demonstrated to reduce *PPP2R1B* mRNA expression in the absence of genetic alterations (Kalla et al, 2007). Among B-subunits, *PPP2R2A* (R2A/PR55α) deletions have been found in breast and prostate cancer and primary plasma cell leukemia (Cheng et al, 2011; Curtis et al, 2012; Liu et al, 2008; Mosca et al, 2013). In melanoma reduced expression of R5A/PR61α and R5C/PR61γ B-subunits has been detected (Mannava et al, 2012; Sangodkar et al, 2015). In CRC, promoter hypermethylation of *PPP2R2B* (R2B/PR55β) has been found in >90% of tumor samples, and decreased expression of this B-subunit promotes rapamycin resistance (Muggerud et al, 2010; Tan et al, 2010). On a post-translational level, hyperphosphorylaiton of PP2A Tyr307 and associated reduction in PP2A activity has been found in 31% and 85% of CRC patient samples respectively (Cristobal et al, 2014b). Similar PP2A-C hyperphosphorylation is also reported in 78% of diagnostic stage acute myeloid leukemia (AML) samples (Cristobal et al, 2011).

2.4.4.1. PP2A inhibitory proteins involved in cancer

The mutations or deletions in PP2A subunit genes occur at relatively low frequency. However, overexpression of various PP2A inhibitory proteins is found in several cancers, which are responsible for inhibition of PP2A tumor suppressor function in cancerous cells (Haesen et al, 2012). These PP2A inhibitory proteins are: inhibitor-1 of PP2A (ANP32a or I_1^{PP2A}), SET (I_2^{PP2A}), cancerous inhibitor of PP2A (CIP2A), cAMP-regulated phosphoproteins (ARPP), α-endosulfin (ENSA), type 2A interacting protein (TIP or TIPRL1), and PME-1. The involvement of PME-1 in cancer has been described at length earlier in this review.

ANP32a and SET inhibit PP2A activity by direct binding to the PP2A-C. These proteins also simultaneously activate PP1 to act on certain substrates, suggesting a possible crosstalk between the two major phosphatases (Katayose et al, 2000). Tyrosine phosphorylation of ANP32a

dissociates it from PP2A, resulting in PP2A mediated inhibition of ERK signaling (Yu et al, 2004). ANP32a has pro-apoptotic activity, and some tumor suppressor function, possibly independent of PP2A inhibition (Haesen et al, 2012). Accordingly, reduced expression of ANP32a has been found in prostate and pancreatic cancers, and it associates with apoptosis resistance in non-small cell lung cancer (NSCLC) (Haesen et al, 2012; Hoffarth et al, 2008). ANP32a has been found in a distinct multi-protein complex along with SET. In this complex, SET antagonizes ANP32a-mediated cell death, indicating that oncogenic function of SET can also function via mechanisms other than PP2A inhibition (Hoffarth et al, 2008). Moreover, SET and ANP32a can bind to the histone tails and suppress gene transcription (Kutney et al, 2004). SET expression is high in proliferating and transformed cells which relates to its pro-survival role in cell cycle checkpoint regulation, and Rac-1 dependent cell migration (Haesen et al, 2012). SET overexpression has been detected in hepatoma, choriocarcinoma, and Wilm's tumor (Haesen et al, 2012). At mRNA level, SET overexpression has been reported in testicular and lung cancer, malignant brain tumors, and head and neck squamous cell carcinoma based on the data analysis at oncomine (Westermarck & Hahn, 2008). Moreover, several different types of hematological malignancies including AML, chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL) express very high levels of SET (Haesen et al, 2012; Westermarck & Hahn, 2008). A fusion protein of SET with Nup214 has also been reported to support leukemia progression (Saito et al, 2004).

CIP2A is an oncogenic protein (Junttila et al, 2007; Westermarck & Hahn, 2008), highly expressed in several different cancers including gastric, lung, colon, esophageal, tongue, breast, prostate, ovary, head and neck squamous cell, astrocytic gliomas, and leukemia (Haesen et al, 2012; Khanna et al, 2013b). The frequency of CIP2A overexpression is very high (40-90%) and it correlates with tumor progression and poor patient survival in many of these cancer types. The expression of CIP2A is almost negligible in normal tissues except in testis (Junttila et al, 2007; Ventela et al, 2012). In cancer cells, CIP2A mediated PP2A inhibition promotes stabilization of phosphorylated Myc (Ser62), and activation of the AKT signaling (Chen et al, 2010; Junttila et al, 2007). Thus, CIP2A contributes to the cancer cell growth and proliferation. Additionally, CIP2A inhibits dephosphorylation of PP2A substrate DAPk (death associated protein kinase), which results in inhibition of cancer cell apoptosis (Guenebeaud et al, 2010). Furthermore, a feedback signaling between E2F1 and CIP2A exits in breast cancer cells, which prevents PP2A mediated growth arrest and senescence (Laine et al, 2013). CIP2A expression in cancer cells is also regulated by active EGFR/MEK/ETS1 pathway and DNA damage checkpoint kinase (CHK1) (Khanna et al, 2013a; Khanna et al, 2013b).

ARPP-16 and -19, which are splice variants of the same gene, and ENSA belong the same family of cAMP regulated phosphoproteins (Dulubova et al, 2001; Haesen et al, 2012; Heron et al, 1998). The binding of ARPP and ENSA to PP2A requires phosphorylation of specific serine residues on these inhibitory proteins. This phosphorylation is carried out by a mitotic kinase Greatwall (MASTL) (Gharbi-Ayachi et al, 2010; Mochida et al, 2010). ENSA or ARPP are also described as mitotic PP2A inhibitors, wherein, their binding inhibits PP2A activity towards CDK1-phosphorylated substrates and promotes G2/M transition. In mammalian cells, R2A/PR55α and R2D/PR55δ containing PP2A complexes are the major targets of these inhibitory proteins (Haesen et al, 2012). Interestingly, ENSA was found to be dysregulated in liver and breast cancers in opposite ways (Chen et al, 2013). High expression of ENSA was detected in liver cancer cell lines and promoter hypomethylation (indicative of active expression) in the liver cancer patients. Whereas in breast cancer, ENSA promoter hypermethylation (indicative of repressed expression) has been detected (Chen et al, 2013). The functional study however suggested a tumor suppressive role of ENSA in liver cancer cells (Chen et al, 2013). Nevertheless, ARPP-19 seems to play the expected oncogenic role in hepatocellular carcinoma (HCC) by inhibiting PP2A's cell cycle arrest function and thereby promoting cell proliferation (Song et al, 2015). In this study, high ARPP-19 was detected in HCC and its expression correlated with the tumor size.

TIPRL1 (or TIP) binding to PP2A has some possible role in the PP2A biogenesis (as previously described). TIPRL1 is involved in regulating PP2A activity towards ATM/ATR mediated DNA damage response and possibly other stress conditions (McConnell et al, 2007). TIPRL1 overexpression has been found in HCC (Song et al, 2012). Its expression in HCC cells promotes binding of PP2A-C with mitogen activated protein kinase kinase 7 (MKK7), affecting the MKK7/JNK signaling, and inhibiting TRAIL-mediated apoptosis (Song et al, 2012).

2.4.4.2. PP2A in gliomas

2.4.4.2.1. Expression of PP2A subunits in human gliomas

The PP2A-A has been reported to be genetically intact in a panel of brain tumors (n=58), especially GBM (Colella et al, 2001). Interestingly, in the same patient panel, the Aα subunit protein expression was 10-fold lower in ~40% of GBM and oligodendroglioma tumors, whereas the Cα and R2A/PR55α expression was unaltered. Hereby, some transcriptional, translational or post-translational mechanisms can be suspected to regulate Aα expression (as mentioned earlier) (Kalla et al, 2007). The expression of PP2A-Aα, Cα and R2/PR55 subunit family and the phosphatase activity has been studied in another panel of malignant

glioma (n=65) patient samples (Gursel et al, 2015; Hofstetter et al, 2012). The expression of all three PP2A subunits in this GBM panel was significantly lower (one-third) compared to non-neoplastic brain tissue samples (Hofstetter et al, 2012). The author observed that the term 'PP2A activity assay' used by Hofstetter et al is incorrect, because according to the experimental procedure mentioned in the article, the activity assay used in this study measures the total Ser/Thr phosphatase activity which also includes the activity of phosphatases other than PP2A, such as PP1, PP4 and PP6. This Ser/Thr phosphatase activity was variable in all samples, with a tendency towards lower activity in GBM compared to normal tissue (Hofstetter et al, 2012). These results are in line with the tumor suppressor function of PP2A. Contradictorily, the higher Ser/Thr phosphatase activity was associated with poor prognosis in this GBM panel (Hofstetter et al, 2012). Additionally, higher PP2A-C mRNA expression was related to worse survival in a panel of primary GBM patients (n=197) from TCGA database (Hofstetter et al, 2012). Among B-subunits, abundant expression of R2C/PR55γ is detected in the normal brain tissues and astrocytes (Fan et al, 2013). However, its expression was found to be downregulated in GBM cell lines as well as in astrocytic glioma patient samples. Moreover, the reduction in R2C expression correlated with the increasing malignancy of these glioma tumors (Fan et al, 2013).

2.4.4.2.2. Tumor suppressor function of PP2A in human gliomas

PP2A-mediated regulation of cell cycle checkpoints, cell proliferation inhibition, and loss of its expression or activity in malignant cells including gliomas have established PP2A as a tumor suppressor. An intermediate filament (IF) protein, synemin, highly expressed in GBM, has been shown to bind and inhibit PP2A(R2/PR55) complexes (Pitre et al, 2012). Synemin knockdown in glioma cell lines enhanced PP2A(R2/PR55) activity. This resulted in dephosphorylation and inhibition of AKT, increased p21 and p27 expression, reduced pRb phosphorylation, G1/S phase cell cycle arrest and inhibition of cell proliferation (Pitre et al, 2012). The synemin-mediated PP2A inhibition might be an alternative mechanism by which AKT activity is maintained specifically in GBM, as synamin expression is scarcely detected in other tumor types (Pitre et al, 2012). In another study, a member of R2/PR55 family of PP2A B-subunits, R2C/PR55γ overexpression was used to identify S6K as its potential target in human glioma cell lines (Fan et al, 2013). R2C/PR55γ overexpression reduced S6K phosphorylation and inhibited glioma cell proliferation *in vitro* and tumor growth in subcutaneous (s.c.) xenograft mouse *in vivo*. Thus, PP2A(R2/PR55) has tumor suppressive function in human gliomas. Another PP2A regulatory subunit from a different family, R5C/PR61γ plays an important role in maintaining normal G0 phase and quiescence (Naetar et al, 2014). In T98G glioblastoma cells, PP2A(R5C/PR61γ) inhibits Ras/Myc signaling and lowers cyclin E level in G2 phase, allowing normal

G0 phase in the next cell cycle. It has been proposed that PP2A(R5C/PR61γ) inhibition in G2-phase is one mechanism by which cancer cells escape G0 in the presence of functionally active p53. Thus, PP2A inhibition in cancer cells might promote G0-escape and shorten the G1-phase (referred to as G1-overdrive), thereby promoting cell proliferation and even drug resistance (Naetar et al, 2014).

2.4.4.2.3. PP2A inhibition as an anti-glioma therapeutic strategy

Against all odds, the pharmacological inhibition of widely established tumor suppressor PP2A leads to cancer cell death, hence proposing pharmacological PP2A inhibitors as anti-cancer agents (Janssens & Rebollo, 2012; Kalev & Sablina, 2011). PP2A inhibition by OA or norcantharidin analogues having higher selectivity for PP2A (LB1 or LB100 and LB1.2 or LB102), leads to glioma cell death via mitotic catastrophe (Castigli et al, 2006; Lu et al, 2009). The mechanism of PP2A-inhibition mediated glioma cell death is attributed to 1) the activation of cell growth promoting AKT and ERK signaling, and 2) impairment of cell cycle checkpoints, forcing improper cell cycle progression resulting in mitotic catastrophe (Gordon et al, 2015; Lu et al, 2009; Lu et al, 2010). Therefore, the anti-cancer function of PP2A inhibitor therapies is based on the normal physiological function of PP2A in cell cycle regulation (Kurimchak & Grana, 2015). The increased AKT phosphorylation associated with LB1.2 treatment was shown to increase phosphorylation of polo-like kinase 1 (PLK1) and MDM2 (Lu et al, 2009). The active PLK1 and MDM2 subsequently inhibit p53 and block cell cycle arrest. The expression of an anti-apoptotic microtubule stabilizing protein TCTP was downregulated upon LB1.2 treatment, possibly contributing to the induction of apoptosis independent of the AKT signaling (Lu et al, 2009). PP2A inhibition by LB1 treatment also enhances the phosphorylation, nuclear export and degradation of NCoR (via increased phospho-AKT). This results in derepression of differentiation promoting genes regulated by nuclear NCoR/HDAC complex (Lu et al, 2010). In this regard, *in vitro* or *in vivo* LB1 treatment in glioma cells enhanced acetylation of histones H3 and H4, and induced the expression of differentiated cell marker GFAP. LB1 and LB1.2 treatments have been examined in s.c. U87MG or U251 human glioma tumor xenograft mouse models (Gordon et al, 2015; Lu et al, 2009; Lu et al, 2010). The treatment with LB1 alone showed slight but significant tumor growth reduction *in vivo*. Interestingly, LB1.2 in combination with TMZ showed drastic regression of s.c. U87MG tumors, with 50% of the animals undergoing complete regression and no tumor regrowth (Lu et al, 2009). Similar tumor growth delay and improvement in mice survival was also observed when LB1 treatment was combined with radiotherapy (Gordon et al, 2015). Thus, the unregulated cell cycle progression by PP2A inhibition works in conjunction with the DNA damaging agents to enhance cytotoxicity in cancer cells. The LB1

doses used alone or in combination with TMZ have shown no toxicity in mice, clearing the grounds for further *in vivo* pre-clinical and clinical testing of this compound (Lu et al, 2009; Lu et al, 2010). Even though there has been an indication that LB1 penetrates BBB, nevertheless, the *in vivo* studies using orthotopic intracranial mouse models are needed to identify whether the non-toxic dosage of LB1 would also be effective for tumor regression in the correct anatomical location. In one study, PP2A has also been linked with casein kinase 2 (CK2)-regulated dephosphorylation of STAT3 (Ser727), induction of its oncogenic transcriptional activity, and enhanced tumor growth in C6 rat glioma xenograft model (Mandal et al, 2014).

It is noteworthy that PP2A inhibition can give rise to resistant tumor cell clones, due to accumulation or acquisition of genetic alterations in the absence of active DNA repair mechanisms and cell cycle checkpoint regulation (Kalev & Sablina, 2011; Naetar et al, 2014). Thus, long term PP2A inhibitor treatment can be potentially carcinogenic. It is also possible that PP2A inhibition (as anti-glioma strategy) might promote the onset of other brain diseases such as Alzheimer's disease. Studies from more than a decade have established PP2A dysfunction as a major underlying factor in Alzheimer's disease (Sontag & Sontag, 2014). Chronically reduced neuron-specific PP2A activity in a transgenic mouse model promotes ERK and JNK pathway activities associated with increased tau-phosphorylation, typical of Alzheimer's disease (Kins et al, 2003).

2.4.4.2.4. PP2A function in glioma stem-like cells (GSCs)

GSCs are generally characterized as a pool of slow growing, undifferentiated, therapy resistant cells that can lead to tumor recurrence (described earlier in this review). PP2A inhibition by LB1 treatment in nestin-positive GSCs induced the expression of GFAP (differentiated cell marker) (Lu et al, 2010). Therefore, PP2A inhibition might inhibit the growth of GSCs by promoting their differentiation. GSCs are usually located at the boundary of hypoxic and necrotic tumor core, and these cells seem to survive the growth-suppressive conditions better than the differentiated tumor cells (Chen et al, 2012). In another GSC cell line, PP2A activity was associated with reduced metabolic activity, G1/S phase growth arrest, and enhanced cell survival under hypoxia (Hofstetter et al, 2012). PP2A inhibition showed two-fold increase in GSC apoptosis under hypoxia, whereas no difference was observed under normoxic conditions. Interestingly, in this cell line under hypoxia, even though PME-1 knockdown induced a potent G1/S phase growth arrest, only a minor reduction in apoptosis was observed, suggesting that PME-1 regulated PP2A may function differently in the regulation of cell cycle and apoptosis (Hofstetter et al, 2012). In addition to hypoxia, nutrient starvation is another tumor microenvironment

factor in which GSCs have a growth advantage (Chen et al, 2012). Here, the PP2A B-subunit R2C/PR55γ overexpression has been reported to promote GBM cell growth under glucose starvation (Li et al, 2015). R2C/PR55γ was shown to act by binding and stabilizing SIK2, thereby forming PP2A-R2C-SIK2 complex, which dephosphorylates and inhibits S6K. However, the significance of this function of PP2A as anti-GSC therapy is uncertain, considering that expression of R2C/PR55γ is often downregulated in human gliomas (Fan et al, 2013; Li et al, 2015).

The crosstalk between PP2A and GSK-3β has been under investigation for several years. Recently, it has been indicated that PP2A-mediated GSK-3β dephosphorylation at Ser9 is required for its pro-apoptotic function in GSCs (Gursel et al, 2015). The inhibition of GSK-3b Ser9 phosphorylation, by using EGFR/AKT signaling inhibitors or PTEN overexpression, was demonstrated to inhibit GSC proliferation with concomitant increase in apoptosis. However, the functional analysis of PP2A-mediated regulation of GSK-3β in GSCs was missing in this study (Gursel et al, 2015).

2.4.5. PP2A reactivation as cancer therapy

Since in cancer cells the PP2A phosphatase activity is inhibited by default, it is reasonable to think that its reactivation might normalize the altered phosphoprotein signaling, and regain the state of homeostasis typical of a normal cell. Therefore, several strategies to reactivate (or activate) PP2A function in the cancer cells have been under investigation with expected cancer cell growth inhibition and death promoting consequences. Herein, one strategy is to use pharmacological compounds, which directly activate PP2A by allosteric binding, and the second strategy is to inhibit the endogenous PP2A inhibitory proteins and thereby achieve PP2A activation indirectly. The potential of PP2A reactivation strategies has not been explored in human gliomas till date; therefore this section will broadly cover the PP2A reactivation in all cancer types.

Although some compounds have been suggested to directly activate PP2A, there is not enough data to support this idea. However, anti-psychotic drugs of the family phenothiazine, including perphenazine and thioredazine, were recently shown to bind PP2A-Aα subunit, resulting in PP2A activation in T-ALL (Gutierrez et al, 2014). A phase-1 clinical trial has been initiated to evaluate the safety of thioridazine in AML patients (www.clinicaltrilas.gov). Additionally, a diterpene antibiotic, forskolin, activates PP2A by reducing inhibitory Tyr307 phosphorylation on the PP2A-C subunit (Cristobal et al, 2011). Forskolin treatment inhibited cell proliferation, altered AKT and ERK phosphorylation, and induced apoptosis in CRC and AML cells (Cristobal et al, 2011; Cristobal et al, 2014b). Synergistic cancer cell cytotoxicity was seen with forskolin in combination with chemotherapy compounds in both CRC and AML (Cristobal et al, 2011; Cristobal et al, 2014b). Another compound, sodium

selenate has been shown to activate PP2A, resulting in increased association between PP2A and tau-protein (Corcoran et al, 2010b; van Eersel et al, 2010). Selenate treatment reduces tau-hyperphosphorylation, stabilizes the neurofibrillary tangles, and improves neuronal symptoms in Alzheimer's disease mouse models (Corcoran et al, 2010b; van Eersel et al, 2010). Selenate treatment was well tolerated in a phase-1 trial in prostate cancer patients with efficacy similar to other anti-angiogenic therapies (Corcoran et al, 2010a). The mechanism of PP2A activation by selenate and its activity in cancer models needs further studies.

Highlighting the role of second PP2A reactivation strategy, a SET inhibitor compound FTY720 (fingolimod) has gained a lot of attention as anti-leukemia agent. FTY720 binding to SET (PP2A inhibitor protein), promotes dissociation of SET from the PP2A holoenzyme complex, and enhances the PP2A activity towards its substrates such as AKT, ERK, STAT5, JAK2 and c-KIT receptor (Perrotti & Neviani, 2013). The anti-cancer activity of FTY720 has been found in c-KIT mutant AML, tyrosine kinase inhibitor resistant CML, multiple myeloma, CRC, HCC and breast cancers (Cristobal et al, 2014a; Neviani et al, 2013; Rincon et al, 2015; Roberts et al, 2010; Sangodkar et al, 2015). FTY720 treatment also induced apoptosis in glioblastoma stem cells, reduced tumor growth in intracranial GSC xenograft mouse model, and improved mice survival in combination with TMZ (Estrada-Bernal et al, 2012). Since FTY720 also functions as an immunosuppressant by modulating sphingosine-1-phosphate receptor (S1PR) internalization, its activity in cancer cells cannot be solely attributed to the PP2A reactivation (Brinkmann et al, 2002). Nevertheless, due to the BBB-penetrable properties and demonstrated activity in glioma mouse model, FTY720 is a potential GBM therapy candidate (Estrada-Bernal et al, 2012). Interestingly, apolipoprotein-E mimetic peptides, COG1410, COG112 and OP449, have been shown to bind and inhibit SET protein, resulting in derepression of PP2A and other SET target proteins such as Rac1 (Agarwal et al, 2014; Christensen et al, 2011; Switzer et al, 2011). SET inhibition with COG112 promotes PP2A phosphatase activity towards AKT and Myc proteins, and inhibits the migration and invasion properties of U87MG GBM cells and a breast cancer cell line (Switzer et al, 2011). Treatment with OP449 inhibits the growth of BCR-ABL1 mutant CML cells, and enhances the cytotoxic effects of TKis in these leukemic cells (Agarwal et al, 2014).

A number of compounds extracted from Traditional Chinese Medicinal herbs, such as celastrol (tripterine), ethoxysanguinarine (ESG) and rabdocoetsin-B inhibit CIP2A either by promoting proteasome-mediated degradation or via downregulation of its expression (Liu et al, 2014a; Liu et al, 2014b; Ma et al, 2011). Moreover, a proteasome inhibitor bortezomib also inhibits CIP2A expression in HCC cells (Chen et al, 2010). The PP2A

reactivation via CIP2A inhibition with these compounds has been demonstrated to inhibit AKT signaling and/or Myc expression, and cell proliferation in lung cancer and HCC, with emerging indications in other cancer types as well (Chen et al, 2010; Liu et al, 2014a; Ma et al, 2011).

Since PP2A-C Leu309 methylation promotes the recruitment of certain R2/PR55 and R5/PR61 family B-subunits, it may be required for tumor suppressor function of PP2A. Therefore, increased PP2A-C methylation might promote the activation of those selective PP2A complexes in the cancer cells. In line with this notion, increased PP2A activity associated with increased PP2A-C Leu309 methylation has been observed with xylulose-5-phosphate (X5P), and a DNA methylating chemotherapy drug chloroethylnitrosourea (CENU) in B16 mouse melanoma cells (Guenin et al, 2008). X5P and CENU treatment in melanoma cells not only induced PP2A activity, but also the PTEN activity, possibly cooperating in the inhibition of AKT activity and Myc expression (Guenin et al, 2008). Another strategy to alter PP2A-C methylation is by inhibition of PP2A methylesterase enzyme PME-1. PME-1 inhibitors, ABL127 and AMZ30, reduce the demethylated PP2A-C level, and inhibit the growth of certain cancer cell lines (Bachovchin et al, 2011a; Bachovchin et al, 2011b; Xia et al, 2015). However, given that PME-1 also inhibits PP2A by direct binding, inhibitors capable of disrupting the PME-1/PP2A binding might be useful as potential PP2A reactivation agents.

2.4.6. PP2A targets in cancer

Several cellular phosphoproteins are direct dephosphorylation targets of distinct PP2A(B-subunit) complexes (as described earlier) (Eichhorn et al, 2009). Phosphorylation of a target protein can affect its activity, stability (therefore expression), sub-cellular localization, interaction with other proteins or biological molecules, or make them substrates for other post-translational modifications. The list of known PP2A targets is very long, and is continually growing. The key PP2A regulated pathways are: MAPK/ERK or JNK, PI3K/AKT/mTOR, WNT/β-Catenin/GSK-3β, Myc, NFkB, ATM/ATR/CHK1/2 (DNA damage response), p53, pRb (cell cycle checkpoints), p70-S6K (protein translational), and PKC (Sablina et al, 2010; Westermarck & Hahn, 2008). Additionally, PP2A also participates in the metabolism, cell differentiation and death signaling, and chromatin modifications. The Ras/MAPK/ERK and PI3K/AKT pathways are among the most well studied PP2A targets in cancer. PP2A inhibition can result in constitutive activation of a number of MAPK pathways, resulting in increased cancer cell proliferation and survival (Junttila et al, 2008). As mentioned earlier, PME-1 inhibits PP2A phosphatase activity towards Ras/MAPK/ERK pathway in human gliomas (Puustinen et al, 2009). However, this thesis study identified another PP2A-target, histone

deacetylase 4 (HDAC4), as a PME-1 regulated protein. The function of these deacetylase enzymes is described in the following section in detail.

2.5. Histone deacetylases

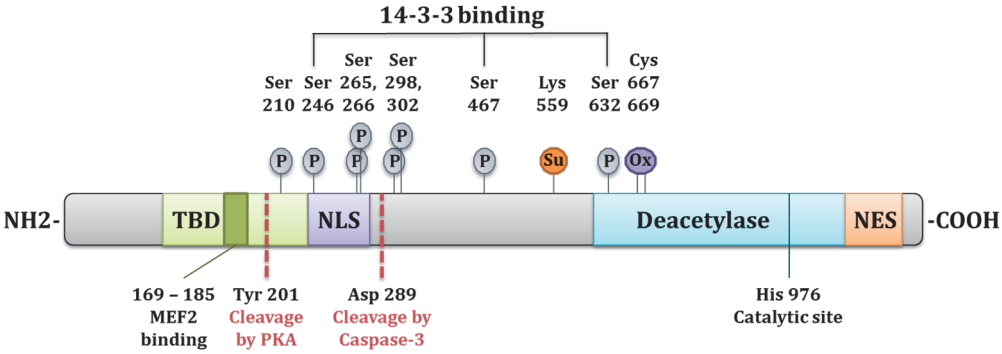
As can be predicted by their name, histone deacetylases (HDACs) are enzymes, which catalyze the removal of acetyl group from conserved lysine (Lys) residues on the nuclear histone proteins. The addition of acetyl group to these proteins is carried out by histone acetyltransferases (HATs). However, following the initial discovery of HATs and HDACs as histone modifiers, several non-histone target proteins have been found in the nucleus and in the cytoplasm. Therefore, based on their enzymatic activity lysine deacetylase (KDAC) name has been suggested. Several HDAC members can shuttle between cytoplasm and nucleus to execute their special functions. Interestingly, HDACs of one family (class II) are thought to play an equally important function in the cytoplasm as in the nucleus.

2.5.1. HDAC classification

Mammalian HDACs can be classified into four categories based on their functional domains and sequence homology to the yeast enzymes: class I (HDAC 1, 2, 3 and 8), class II (HDAC 4, 5, 6, 7, 9 and 10), class III (Sirt 1-7), and class IV (HDAC 11) (de Ruijter et al, 2003; Witt et al, 2009). The catalytic activities of class I, II and IV HDACs depend on the presence of Zn^{2+} ions, for which they are regarded as classical HDACs (de Ruijter et al, 2003). One exception to this category is HDAC8, which uses mainly Fe^{2+} ions in addition to Zn^{2+} (Gantt et al, 2006). The class III HDACs (Sirtuins) use nicotinamide adenine dinucleotide (NAD+) as a catalytic cofactor (Kleszcz et al, 2015). Due to a different catalytic mechanism, classical HDAC inhibitors such as trichostatin A (TSA) cannot inhibit class III HDACs. Based on the additional domains and sub-cellular localization, class II HDACs are further subdivided into class IIa (HDAC 4,5, 7 and 9) and class IIb (HDAC 6 and 10). The class I HDACs are exclusively nuclear, except for HDAC3, which can be found in the cytoplasm, even though it is also mainly nuclear (de Ruijter et al, 2003).

The class II HDACs differ from class I in the respect that the class IIa HDACs can efficiently shuttle between the nucleus and cytoplasm and have putative targets in both compartments, and the class IIb HDACs are mainly cytoplasmic (Lee et al, 2015b). Additionally, class II HDACs (especially class IIa) differ from class I for having low deacetylase enzymatic activity, absence of DNA binding domain, and presence of a long N-terminal adaptor domain, which promotes the association of class II HDACs with DNA binding proteins, and/or serves other atypical functions (Martin et al, 2007; Spiegel et al, 2012). Among class IIb, the

main function of HDAC6 is deacetylation of α-tubulin and HSP90 in the cytoplasm (Witt et al, 2009). The class IIa HDACs contain a C-terminal deacetylase domain with a catalytic histidine residue (His976 in HDAC4) instead of catalytic tyrosine residue (present in the class I HDACs) (Figure-5). This difference seems to be responsible for the low deacetylase activity of class IIa enzymes (Clocchiatti et al, 2011). However, class IIa HDACs can recruit a multi-protein HDAC3/NCoR/SMRT transcriptional co-repressor complex to exert their deacetylase function indirectly (Clocchiatti et al, 2013; Fitzsimons, 2015). Moreover, an additional zinc-binding domain located near the active site possibly provides substrate specificity to the class IIa HDACs (Bottomley et al, 2008).



Site	Phosphorylating kinase/s
Ser 246	CaMK-I, IV, AMPK, PKD1, SIK1/2
Ser 467	CaMK-I, II, IV, PKD1, SIK1/2, AMPK?
Ser 632	CaMK-II, PKD1, SIK2
Ser 210	CaMK-IIB
Ser 265/266	PKA, DIRK1B, AURKB
Ser 298*/ 302	GSK-3β

14-3-3

P Phosphorylation site
Su SUMOylation site
Ox Oxidation site

* site possibly dephosphorylated by PP2A

Figure-5. Illustration of HDAC4 structural domains and location of known post-translational modifications. *The table depicts the kinases that have been shown to phosphorylate HDAC4 in some cellular models. TBD: transcription factor binding domain (contains MEF2 and other transcription factor binding sites); NLS: nuclear localization signal (244 – 279); Deacetylase domain (621 – 1039); NES: nuclear export signal (1044 – 1069). This figure is based on the information provided by previously published articles (Backs et al, 2011; Di Giorgio & Brancolini, 2016; Fitzsimons, 2015; Mielcarek et al, 2015; Wang & Yang, 2001).*

2.5.2. HDAC4 function and regulation

In the nucleus, the recruitment of class IIa HDACs by certain DNA-binding transcription factors is responsible for histone deacetylation, and

transcriptional repression (Figure-6) (Clocchiatti et al, 2011). Transcription factors of the myocyte enhancer factor-2 (MEF2) family, MEF2A, -C and -D, are the best-characterized examples of HDAC4 targets. HDAC4 leads to transcriptional repression of several MEF2-target genes via histone deacetylation, though it is not clear whether it can occur independently of the HDAC3/SMRT/NCoR complex (Figure-6) (Clocchiatti et al, 2011; Martin et al, 2007; Mielcarek et al, 2015). Moreover, HDAC4 (and other class IIa HDACs) can inhibit MEF2 activity by other mechanisms involving their N-terminal domain, and independent of their deacetylase (C-terminal domain) activity (Figure-5). For instance, HDAC4 N-terminal domain can associate with an adaptor protein, heterochromatin protein 1 (HP1), which further recruits a histone methytransferase (HMT), SUV39H1 (Zhang et al, 2002). Since histone methylation and deacetylation are the epigenetic marks representative of transcriptionally repressed chromatin, the methylation (by SUV39H1) of histones coupled with histone deacetylation carried out by HDAC4 further represses the transcription of MEF2-target genes (Figure-6) (Zhang et al, 2002).

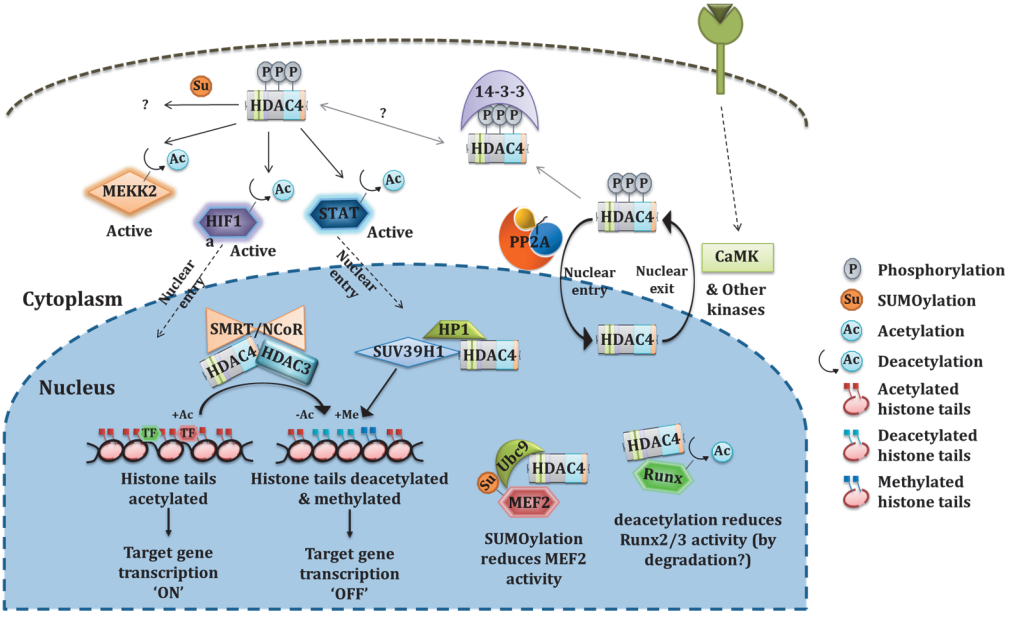


Figure-6. HDAC4 localization and possible functions in the intracellular compartments. *HDAC4 nucleo-cytoplasmic shuttling is regulated by phosphorylation. In the nucleus, HDAC4 can participate in various complexes, ultimately leading to repression of genes regulated by specific transcription factors (only MEF2 and Runx are shown in this simplified view). In the cytoplasm, 14-3-3 binding sequesters phosphorylated HDAC4. Either 14-3-3-bound or free HDAC4 in the cytoplasm can deacetylate certain target proteins (MEKK2, HIF1a, and STAT1 are shown). Possible SUMOylation targets of HDAC4 in the cytoplasm are not yet known.*

Surprisingly, HDAC4 is also reported to act as a SUMO E3 ligase. The HDAC4 N-terminal domain promotes MEF2 SUMOylation by recruiting small ubiquitin-like modifier enzymes, SUMO2 and SUMO3, and the SUMO-conjugating enzyme Ubc9 (Figure-6) (Gregoire & Yang, 2005). The MEF2 SUMOylation decreases its transcriptional activity. Surprisingly, SUMOylation of HDAC4 itself inhibits its function of promoting MEF2 SUMOylation (Gregoire & Yang, 2005).

Another important HDAC4 target is Runt-related transcription factor (Runx) family of transcription factors (Clocchiatti et al, 2011; Martin et al, 2007; Mielcarek et al, 2015). Similar to MEF2, HDAC4-mediated inhibition of Runx also involves different mechanisms: 1) via its N-terminal domain HDAC4 interferes with the binding of Runx2 to its target promoters, 2) HDAC4 can also repress the *RUNX2* gene transcription, 3) HDAC4 directly deacetylates Runx2 and Runx3 leading to their degradation and target-gene transcription inhibition. Thus, Runx2 is also a non-histone HDAC4 deacetylation target (Figure-6).

Apart from these, serum response factor (SRF), yin-yang 1 (YY1), forkhead box P3 (FOXP3), nuclear factor of activated T-cells (NFAT1/3), signal transducer and activator of transcription (STAT1), and hypoxia inducible factor (HIF1α) transcription factors, and nuclear receptors such as estrogen and androgen receptors (ER and AR) are also regulated by HDAC4 either by direct binding or via adaptor proteins and/or deacetylation by HDAC4 co-repressor complex (Clocchiatti et al, 2013; Davis et al, 2003; Kosiorek et al, 2014; Liu et al, 2009; Reddy et al, 2012). HDAC4 can also promote SUMOylation of some of its interacting partners such as Liver X receptor (LXRα and β), MEF2, AR, and even sirtuin SIRT1 (class III HDAC) (Gregoire & Yang, 2005; Han et al, 2016; Lee et al, 2009; Yang et al, 2011). Another unconventional function of HDAC4 relates to its role in DNA damage response pathway. Following DNA damage, HDAC4 translocates to the nucleus where it binds acetylated-p53 and NF-Y bound to the G2/M promoters, inhibits the transcription of their target genes, and promotes G2/M cell cycle arrest (Basile et al, 2006; Martin et al, 2007).

The HDAC4 targets in the cytoplasm have started to be uncovered. These targets can be regulated by HDAC4 deacetylase or SUMOylation function (Clocchiatti et al, 2013). Using exogenous FLAG-tagged HDAC4 in HEK293 cells, α- and β-tubulin, kinesin-like protein KIF11, α-spectrin, heat shock factor HSP70, α-actinin 4, and TBLR1 proteins have been identified as cytosolic interacting partners of HDAC4 (Paroni et al, 2008). Interestingly, the deacetylation of transcription factors HIF1α and STAT1 by HDAC4 is also reported to occur in the cytoplasm, in HDAC3-independent manner (Figure-6) (Clocchiatti et al, 2013). Additionally, HDAC4 can also deacetylate and activate MEKK2 in the cytoplasm

(Figure-6), resulting in the activation of downstream AP1 transcription activity involved in neurogenic muscle atrophy (Choi et al, 2012).

2.5.2.1. HDAC4 sub-cellular localization and regulation by phosphorylation

Since HDAC4 has important roles in both cytoplasm and the nucleus, its nucleo-cytoplasmic shutting needs to be under tight control (Di Giorgio & Brancolini, 2016). Structurally, HDAC4 contains a nuclear localization signal (NLS) present in its N-terminal domain, and a nuclear export signal (NES) in the C-terminal domain (Wang & Yang, 2001). HDAC4 nuclear export is facilitated by its phosphorylation at the conserved serine residues Ser246, Ser467 and Ser623 (Figure-5) and nuclear transporter exportin 1 (CRM-1) (Fitzsimons, 2015). These phosphoserine residues mediate HDAC4 binding with a chaperon protein 14-3-3 dimer. This binding promotes conformational changes in HDAC4, exposing the NES required for nuclear export, and its sequestration in the cytoplasm by masking the NLS required for its nuclear import (Clocchiatti et al, 2011; Di Giorgio & Brancolini, 2016; Nishino et al, 2008). This relives the transcription factors (like MEF2) from HDAC4-mediated repression and results in derepression of the target genes. Moreover, this shuttling allows HDAC4 to act on its cytosolic targets.

The phosphorylation of HDAC4 at these serine residues is carried out by a number of kinases which function as a bridge between extracellular stimuli and the epigenetic regulators in the nucleus (Figure-5) (Parra & Verdin, 2010). Some kinases display preference for distinct class IIa HDACs and sometimes even for the specific serine residue of a HDAC (Martin et al, 2007). Nevertheless, majority of the kinases which phosphorylate HDAC4 (and even other class IIa HDACs) belong to the CaMK superfamily (Martin et al, 2007). HDAC4 is a specific target for CaMK-II (Ser467 and 632). CaMK-I and -IV can phosphorylate HDAC4 at Ser246 and 467 residues similar to other class IIa HDACs (Di Giorgio & Brancolini, 2016; Parra & Verdin, 2010). Additionally, other members of the CaMK superfamily such as protein kinase D1 (PKD1), microtubule affinity regulating kinase (MARK), AMPK, SIK1, -2 and -3 can phosphorylate HDAC4 at one or more of the three serine residues required for 14-3-3 binding and cytosolic accumulation (Di Giorgio & Brancolini, 2016). Apart from the 14-3-3 binding serine residues, several other phosphorylation sites have been reported for HDAC4 (Figure-5) (Clocchiatti et al, 2013).

The phosphorylated HDAC4 is in turn a substrate for protein phosphatases. The identity of HDAC4 phosphatases, especially those involved in dephosphorylation of 14-3-3 binding phosphoserine residues, is obscure. However, few reports have suggested the

involvement of PP2A, and possibly PP1 in HDAC4 nuclear translocation that indirectly relates to its dephosphorylation (Brush et al, 2004; Han et al, 2016). Forskolin, a nonspecific PP2A activator, dephosphorylates exogenous Flag-tagged HDAC4 at Ser246, and promotes its nuclear translocation in chondrocytes (Kozhemyakina et al, 2009). PP2A silencing inhibits the forskolin-induced HDAC4-Flag nuclear import in these cells. Evidence for direct dephosphorylation of HDAC4 by PP2A has been shown using *in vitro* assays, or shifts in the electrophoretic mobility of HDAC4 upon treatment with PP2A inhibitor OA (Paroni et al, 2008). Specific HDAC4 phosphosites dephosphorylated by PP2A are not yet known. Nevertheless, PP2A has been shown to promote HDAC4 nuclear import (Illi et al, 2008; Kozhemyakina et al, 2009; Paroni et al, 2008). Surprisingly, dephosphorylation of exogenously expressed HDAC4 at Ser298 (Figure-5), a site different from 14-3-3 binding Ser residues, efficiently drives it to the nucleus (Paroni et al, 2008). This site is possibly dephosphorylated by PP2A (Paroni et al, 2008). Moreover, HDAC4 interaction with PP2A(R2A/PR55α) complexes has been found in HEK293 cells, ATM KO mouse brain and human ataxia telangiectasia brain tissues (Li et al, 2012; Paroni et al, 2008). Exogenously expressed HDAC4 is also targeted by PP2A(R5A/PR61α) complexes in HeLa cells (Cadot et al, 2009). The exact mechanism of HDAC4 dephosphorylation and its nuclear import is still open for investigation especially at the level of endogenous proteins.

Apart from the 14-3-3 binding serine residues, phosphorylation of additional sites on HDAC4 is associated with variable functions. GSK-3β mediated phosphorylation of HDAC4 at Ser298 serves as a signal for polyubiquitination and proteasome degradation in untransformed cells (Figure-5). This serine residue is dephosphorylated by PP2A, suggesting the possibility that PP2A activity can protect HDAC4 from degradation (Cernotta et al, 2011). HDAC4 is also a substrate for caspase-3 mediated cleavage (Asp289 residue), which generates an N-terminal fragment containing NLS (Figure-5) (Paroni et al, 2004). This N-terminal HDAC4 fragment translocates to the nucleus, represses MEF2C and possibly other transcription factors, and induces apoptosis (Paroni et al, 2004). PKA can also trigger HDAC4 cleavage at residues 201/202 generating a smaller N-terminal fragment (Figure-5). This smaller fragment also translocates to the nucleus, but specifically represses MEF2 transcriptional activity (Backs et al, 2011). Furthermore, HDAC4 subcellular localization and activity is under the control of complex regulatory mechanism involving SUMOylation, oxidation (redox-sensing affected by ROS) and proteolysis (Figure-5) (Han et al, 2016). Several of these regulatory mechanisms can be cell type and stage dependent. Besides, the relevance of several of the above-described regulatory mechanisms in the context of cancerous cells is still lacking.

2.5.3. HDAC4 in cancer

The epigenetic changes implicated in tumorigenesis lead to silencing of tumor suppressor genes (by hypermethylation and/or hypoacetylation), and derepression of oncogenes (by hypomethylation and hyperacetylation) (Bojang & Ramos, 2014). Since different HDACs regulate distinct cellular pathways, it is possible that in cancer cells some HDACs are upregulated or activated whereas some others are downregulated or repressed (Lee et al, 2015b). Moreover, the tissue specific expression and function of HDACs itself, their targets, and even their regulatory proteins makes some HDACs more attractive factors for tumor progression in some cancers than the others.

One study reported the expression of class I, II and IV HDACs in a small panel (n=43) of grade I-IV astrocytic glioma patient samples compared to normal brain tissue samples (Lucio-Eterovic et al, 2008). There was no difference in HDAC4 mRNA expression in normal brain (n=11) and LGG samples (n=20). The grade III glioma samples (n=5) displayed highest HDAC4 mRNA expression (2.5 fold higher than normal brain), whereas in grade IV GBM samples (n=18) its expression dropped even lower than the normal brain and LGG samples (Lucio-Eterovic et al, 2008). Similar reduction in mRNA expression from grade III to GBM samples was observed for HDAC6, -7 and -11. Interestingly, these researchers also observed histone H3 (but not H4) hyperacetylation in GBM compared to normal brain tissue and LGG (Lucio-Eterovic et al, 2008). In a previous study utilizing the AMC Human Transcriptome Map database, HDAC4 expression was undetectable in normal tissue from different organs including brain, whereas it was highly elevated in the cancerous tissues, especially brain, ovary, prostate, pancreatic and colon cancers (de Ruijter et al, 2003). In line with the results from Lucio-Eterovic et al, recently it has been reported that HDAC4 mRNA expression is downregulated in HGGs (grade III and IV) as compared to LGGs (grade II) (Cheng et al, 2015). Moreover, high HDAC4 expression correlates with better survival of glioma patients, as reported in two independent studies (Cheng et al, 2015; Dali-Youcef et al, 2015). Thus, it can be concluded that HDAC4 expression is altered in gliomas, and reduction in its mRNA expression correlates with tumor progression. However, HDAC4 undergoes several PTMs, which regulate its protein expression and activity in different cellular compartments. Therefore, the protein level expression and subcellular localization analysis (for instance by IHC) in human gliomas is highly desirable. Additionally, HDAC4 expression may be a significant contributor to drug resistance. For instance, elevated levels of HDAC4 have been detected in a panel of cisplatin-resistant tumors as compared to the paired treatment-naïve tumors from the ovarian cancer patients (Stronach et al, 2011). Among other cancer

types, overexpression of HDAC4 has been reported in breast and gastric cancers (Colarossi et al, 2014; Ozdag et al, 2006).

Several accumulating functional studies in cancer cells underscore HDAC4 as an anti-cancer target. Given that HDAC4 promotes cell cycle arrest as a component of the DNA damage-induced repair complex, specific HDAC4 inhibitors could have potential use as anti-cancer therapy in combination with radiation or DNA damage inducing chemotherapies (Basile et al, 2006; Clocchiatti et al, 2011). Furthermore, HDAC4 inhibits cell cycle regulator p21 in a p53-independent manner by binding SP1/SP3 and histone deacetylation of the p21 promoter containing binding sites for these transcription factors (Mottet et al, 2009). HDAC4 silencing induces p21 expression and inhibits glioblastoma U87MG cell proliferation *in vitro* and tumor growth *in vivo* in chick chorioallantoic membrane model (Mottet et al, 2009). Notably, HDAC4 knockdown inhibits the growth of p53 expressing cancer cells to some extent; however, in p53-null cells it leads to cell cycle growth arrest and apoptosis (Cadot et al, 2009).

Interestingly, in HCC and lung cancer the lack of microRNAs (miR-1 and miR-22) is responsible for the elevated levels of HDAC4, and silencing HDAC4 inhibits cell growth and sensitizes cancer cells to chemotherapies (Nasser et al, 2008; Zhang et al, 2010). Highlighting the role of its cytosolic targets, HDAC4 binding and deacetylation protects HIF1α from degradation (Geng et al, 2011). Therefore, HDAC4 has a possible role in tumor progression under hypoxic conditions and in the survival of cancer stem cells (CSCs). In NSCLC cells, HDAC inhibitor panobinostat enhances their response to cisplatin, resulting in apoptosis, which is linked with the panobinostat-mediated degradation of HDAC4 and HIF1α (Fischer et al, 2015). Additionally, HDAC4 mediated regulation of STAT1 acetylation has been implicated in cisplatin-resistance in ovarian cancer (Stronach et al, 2011). Several emerging reports have confirmed the role of HDAC4 in drug resistance in breast, gastric, and lung cancer and multiple myeloma, proposing HDAC4 inhibitor and standard chemo/drug-therapy combinations as potent anti-cancer treatment modalities (Chen et al, 2014; Colarossi et al, 2014; Kikuchi et al, 2015; Yu et al, 2013).

2.5.4. HDAC inhibitors as cancer therapy

After the initial discovery of TSA as HDAC inhibitor (HDACi), several different families of compounds have been identified to display similar properties (Mottamal et al, 2015; Yoshida et al, 1990). Many of these HDACi compounds have reportedly displayed anti-cancer activities in a number of cancer cell lines in *in vitro* cell culture and *in vivo* tumor xenograft animal models, and some have paved their way to the clinics (Lee et al, 2015b; Mottamal et al, 2015; Spiegel et al, 2012). Researchers

are still trying to understand the various mechanisms by which different HDAC inhibitors inhibit tumor growth, however, some important mechanisms are now known. Majority of the conventional HDAC inhibitors are active against both class II HDACs, therefore also referred to as pan-HDAC inhibitors, such as TSA, vorinostat (SAHA), panobinostat (LBH589) (Mottamal et al, 2015; Witt et al, 2009). Nevertheless, more potent inhibition of class I HDACs is observed for several of these compounds, which can be referred to as selective class I HDAC inhibitors, for example valproic acid, entinostat, and romidepsin (FK228) (Mottamal et al, 2015; Witt et al, 2009). Only recently selective class IIa HDAC inhibitors have started to emerge, which seems obvious provided the accumulating studies establishing this class of HDACs are potential drug targets (Di Giorgio et al, 2015).

The general mechanism of HDACi-mediated anti-cancer activity involves enhanced histone acetylation of tumor suppressor gene promoters, such as p21, chromatin opening for the binding of specific transcription factors and thereby enhanced tumor suppressor gene expression (which was previously inhibited by overactive HDACs) (Clocchiatti et al, 2011; Richon et al, 2000; Vrana et al, 1999). Additionally, increase in acetylation of non-histone HDAC-target proteins can function by different mechanisms to either induce similar transcription changes or completely different protein activities. For instance, romidepsin treatment enhances p53 acetylation, which protects it from degradation (Zhao et al, 2006). The acetylated and stabilized p53 in turn binds p21 promoter and upregulates its expression (Zhao et al, 2006). Recently, it has been established that the HDAC inhibitor mediated induction of p21 expression not only depends on class I HDACs but also on HDAC4 inhibition (Liu et al, 2009; Mottet et al, 2009; Shen et al, 2016; Wilson et al, 2008). The changes in epigenetic landscape and intracellular signaling upon HDACi treatment eventually results in cell cycle growth arrest, inhibition of cell proliferation and induction of programmed cell death (Di Giorgio et al, 2015; Mottamal et al, 2015). Moreover, some HDAC inhibitors can also inhibit angiogenesis, or promote immune cell mediated tumor cell clearance (Di Giorgio et al, 2015; Turtoi et al, 2015). The HDAC inhibitors approved by the United States FDA are – vorinostat and romidepsin for cutaneous T-cell lymphoma (CTCL), and panobinostat for multiple myeloma (Laubach et al, 2015; Mann et al, 2007; VanderMolen et al, 2011). Among these, the BBB-penetrability of panobinostat is high suggesting this compound may have potential use for treatment of brain tumors (Lee et al, 2015b). Notably, it has already been under investigation in clinical trials for gliomas (Bagcchi, 2015; Drappatz et al, 2012; Lee et al, 2015a). Several other HDACi compounds are under advanced phase clinical trials, and are progressing towards their approval for the treatment of solid tumors and blood cancers (Lee et al, 2015b; Mottamal et al, 2015).

Even though HDAC inhibitors are expected to inhibit the deacetylase activity of target HDACs, reduction in expression of HDAC4 protein has also been detected with some compounds. In line with this notion, panobinostat, vorinostat or TSA treatment displayed reduced HDAC4 protein levels, likely by promoting its SUMOylation, ubiquitination and proteasome-mediated degradation (Du et al, 2015; Fischer et al, 2015; Scognamiglio et al, 2008).

Recent developments to identify class IIa specific inhibitors have provided few compounds with higher selectivity for HDAC4 inhibition. For example, LMK235 inhibits HDAC4 and HDAC5 with IC50 values of 11.9 and 4.2 nanomolar respectively, whereas, the inhibition of class I and IV HDACs was observed only at 25-100 fold higher concentrations (Marek et al, 2013). Another compound MC1568 is a selective class IIa HDACi, as it inhibits class IIa HDAC4, 5 and 6 in the nanomolar range, whereas class I HDACs were not inhibited at these concentrations (Nebbioso et al, 2010). The described class IIa HDAC inhibitors have displayed moderate cancer cell cytotoxicity alone or enhanced cell death in combination with chemotherapy compounds such as docetaxel or cisplatin (Colarossi et al, 2014; Marek et al, 2013). These compounds provide useful tools to dissect the class IIa specific anti-cancer effects, and depending on their pharmacokinetic properties might become potential clinically relevant anti-cancer agents.

3. AIMS OF THE STUDY

PME-1 regulates PP2A activity by demethylating the highly conserved C-terminal tail and by direct binding to the active site of PP2A-C. Specific PP2A complexes and targets regulated by PME-1 remain elusive. Previously, PME-1 expression was shown to correlate with the proliferation and progression of human astrocytic gliomas, however, whether PME-1-mediated PP2A inhibition is involved in GBM therapy resistance is unknown. The expression and clinical relevance of PME-1 in several other cancer types including CRC has also not been studied.

The specific aims of this thesis are as following.

1. Drug screening to identify potential synthetic lethality with PME-1 depletion in GBM
2. To inspect the PME-1 mediated drug resistance mechanisms in GBM
3. To study the *in vivo* efficacy and clinical relevance of PME-1 silencing therapy in GBM
4. To analyze the expression and prognostic significance of PME-1 in CRC

4. MATERIALS AND METHODS

The materials and methods used in this study are tabulated below. The detailed description of all the methods can be found from the original publications.

Methods	Used in publication
Apoptosis (Nuclear fragmentation or sub-G0/G1 phase) analysis by FACS	II
Caspase-3 and -7 activity assay	II
Cell proliferation (WST-1) assay	III
CellTiter-glo (CTG) cell viability assay	II, III
Colony formation assay	I, II
Colony formation assay quantification by ImageJ	I, II
GSC isolation and culture on matrigel	II
Immunofluorescence assay (IFA)	II, III
Immunohistochemical (IHC) staining	II, III
Kaplan-Meier survival analysis	III
Lentivirus infection and shRNA clones	II
Multivariate survival analysis	III
Mouse xenograft	II
PP2A activity assay (PP2A immunoprecipitation phosphatase assay)	II
Proximity Ligation Assay (PLA)	II
siRNA transfection	II, III
TCGA RNAseq data analysis by SAS JMP Pro	III

Materials

Cell lines	Cell type	Used in publication
CW-2	Human colorectal carcinoma	III
E2 (GSC)	Human glioblastoma	II
G7 (GSC)	Human glioblastoma	II
HCA-7	Human colorectal carcinoma	III
HeLa	Human cervical adenocarcinoma	II
MCF-7	Human mammary adenocarcinoma	II
Normal rat astrocytes	Astrocytes isolated from newborn rat brain (hippocampus region)	II

PC3	Human prostate adenocarcinoma	II
R10 (GSC)	Human glioblastoma	II
R15 (GSC)	Human glioblastoma	II
R24 (GSC)	Human glioblastoma	II
SKOV-3	Human ovarian adenocarcinoma	II
T98G	Human glioblastoma	I, II
U251MG	Human glioblastoma	II
U87MG-luciferase	Human glioblastoma	II

Antibodies	**Catalog Number**	**Application**	**Used in publication**
Primary antibodies			
acetylated-lysine proteins	CST#9441	WB	II
Actin	AC-40	WB	II
AKT phosphorylated (Thr 308)	sc-16646	WB	III
BAD	sc-943	WB, IHC	II
b-tubulin	MAB3408	WB	II
CD133/1	W6B3C1	WB	III
CIP2A	sc-80659	WB	II
ERK-1/2 phosphorylated (Thr202/Tyr204)	CST#4370	WB	III
GAPDH	5G4-6C5	WB	II, III
HDAC4	sc-11418	WB, IFA, PLA	II
HDAC4	sc-46672	IHC	II
Histone H3	ab1791	WB	II
c-Myc	ab32072	WB	II
Nestin	ab22035	WB	II
NFkB	CST#8242	WB	II
Olig2	AF2418	WB	II
PERP	ABE606	WB	II
PME-1	sc-20086	WB	II
PME-1	sc-25278	WB, IFA, IHC	II, III
PP2A-C	CST#2038	WB	II
PP2A catalytic α (PP2A-Cα)	BD-610556	PLA	II
SOX2	ab75485	WB	II
Histone H3 acetylated Lys9	H5110-13G	WB	II
Secondary antibodies			
Goat anti-mouse IgG, Alexa-594 conjugated	A-11005	IFA	III

Goat anti-rabbit IgG, Alexa-488 conjugated	A-11008	IFA	II
Goat anti-mouse IgG, HRP conjugated	P0447	WB	II, III
Swine anti-rabbit IgG, HRP conjugated	P0399	WB	II, III

WB: Western blotting; IFA: Immunofluoroscence asaay; IHC: Immunohistochemical staining; PLA: Proximity ligation assay

Inhibitors	**Used in publication**
ABL127 (PME-1 inhibitor)	II
ABT263 (Bcl-2/-XL inhibitor)	II
Arcyriaflavin-A	II
CEP-701 (lestaurtinib)	II
Chelerythrine chloride	II
DHPCC-9 (PIM kinase inhibitor)	II
FTY720	II
GÖ 6976	II
H7	II
H8	II
H89	II
K252a	II
K252c	II
Lapatinib	II
Temozolomide (TMZ)	II
Okadaic acid (OA) (PP2A inhibitor)	II
LY294002 (PI3K inhibitor)	II
Panobinostat (LBH-589)	II
PKC412 (midostaurin)	II
Rebeccamycin	II
RO-31-8220	II
Romidepsin (FK-228)	II
SAHA (Vorinostat)	II
SB218078	II
SB431542 (TGFb-R inhibitor)	II
Sodium Selenate	II
Sunitinib	II
Staurosporine (STS)	I, II
Tandutinib	II
UCN-01	I, II
UO126 (MEK inhibitor)	II
Vandetanib	II
Xylulose-5-phosphate (X5P)	II
Z-VAD-FMK (pan-caspase inhibitor)	II

Reagents	**Used in publication**
Accutase (Invitrogen)	II
B27 supplement (Gibco)	II
BSA – Bovine serum albumin (Sigma-Aldrich)	II, III
Crystal violet (Sigma-Aldrich)	I, II
DAPI (Invitrogen)	II
DMEM - Dulbecco's modified Eagle's medium (Sigma-Aldrich)	II, III
DMEM/F12 advanced medium (Gibco)	II
DMSO (Sigma-Aldrich)	I, II
EGF – Epidermal growth factor (Invitrogen)	II
EMEM - Eagle's minimum essential medium (Sigma-Aldrich)	I, II
FBS – Fetal bovine serum (Gibco)	I, II, III
Formaldehyde (Sigma-Aldrich)	I, II
b-FGF – basic-fibroblast growth factor (Invitrogen)	II
L-glutamine (Invitrogen)	I, II, III
Goat serum (Abcam)	II, III
Heparin (Invitrogen)	II
Hoechst 33342 (Invitrogen)	III
in-vivo jetPEI (Polyplus Transfection)	II
Isoflurane	II
Lipofectamine RNAiMAX (Invitrogen)	II, III
Matrigel (Becton Dickinson)	II
Mowiol (Sigma-Aldrich)	II, III
N2 Supplement (Invitrogen)	II
Paraformaldehyde (PFA) (Sigma-Aldrich)	II, III
Penicillin (Sigma-Aldrich)	I, II, III
PhosSTOP - Phosphatase inhibitor cocktail (Roche)	II, III
Propidium iodide (Sigma-Aldrich)	II
Protease inhibitor cocktail (Roche)	II, III
Puromycin (Sigma-Aldrich)	II
RPMI-1640 - Roswell Park Memorial Institute medium (Sigma-Aldrich)	II, III
Streptomycin (Sigma-Aldrich)	I, II, III
XenoLight D-Luciferin substrate	II

5. RESULTS

5.1. PME-1 knockdown sensitizes GBM cells to multikinase inhibitors (II)

5.1.1. PME-1 knockdown and multikinase inhibitor treatment induces dramatic apoptosis of T98G glioblastoma cells (II)

A library of small-molecule kinase inhibitors was acquired to evaluate their treatment response in PME-1 silenced GBM cells. Selected inhibitors were able to target different members of the protein kinase A, G, C (AGC), Calcium calmodulin kinases (CAMK) or receptor tyrosine kinase (TK) family (Table-2). Additionally, selective inhibitors of MEK and PI3K, and a broad specificity kinase inhibitor staurosporine (STS) were included in the library (Table-2). Treatment responses were analyzed for apoptosis induction measured by sub-G0/G1 phase cell cycle (nuclear fragmentation) analysis. The inhibitor screening displayed marked drug resistance of T98G cells (scrambled siRNA transfected control) (II Figure 1A). However, in cells silenced for PME-1 significant induction of apoptosis was observed with some selective inhibitors such as H7 (PKA/C/G inhibitor) and LY294002 (PI3K inhibitor), and multi-target TK inhibitor sunitinib (II Figure 1A). Remarkably, the most potent apoptosis induction in PME-1 depleted cells was observed with the multi-target kinase inhibitor STS, which was therefore selected for further investigation (II Figure 1A). Non-specific siRNA sequence and transfection effects were excluded (II Figure 1B-D and Supplementary Figure 1A-B). Moreover, the synthetic lethality was specific for GBM cells, and no toxicity was seen in the normal astrocytes isolated from newborn rat brain (II Figure 1I).

Table-2. ***Major target kinases and the respective kinase family targeted by the kinase inhibitors included in the library.***

Inhibitor	Target Kinases	Kinase family
H7	PKC, PKA, PKG	AGC
H8	PKG, PKA, MLCK	AGC/CAMK
H89	PKA, PKG, PKCμ, MSK1, ROCKII	AGC/CAMK
Chelerythrine chloride	PKC	AGC
Sunitinib	PDGFR, KIT VEGFR, FLT3, RET	TK
Tandutinib	FLT3, PDGFR, KIT	TK
Lapatinib	EGFR, ERBB2	TK
Vandetanib	VEGFR2, EGFR	TK
U0126	MEK1/2	STE
LY2940002	PI3K	PKL
Staurosporine (STS)	PKCα/γ/η/δ, FGR (SRC2), PhK, S6K, CDK1,2,4,5, PKA, PKG, SYK, LYN, CaMKII, MLCK, GSK3b, PIM1	AGC/CAMK/ CMGC/TK

Further, a second inhibitor library comprising various STS structural analogs and derivatives was acquired with a focus on those compounds that were already under clinical trials (II Supplementary table 1). These compounds were also tested for apoptosis induction in T98G cells with or without PME-1 knockdown (II Figure 2A and Table 1). Although all these compounds belong to the indolocarbazole family, a varying degree of structural variability was present in them (II Table 1). A varying degree of synthetic lethal response was seen with these compounds in PME-1 silenced T98G cells (II Figure 2A). Nevertheless, UCN-01, PKC412, CEP-701 and K252a displayed robust apoptosis induction, similar to STS (II Figure 2A). Based on the potency of STS analogs in inducing apoptosis in PME-1 silenced cells, a structure-activity relationship (SAR) analysis was performed (II Table 1). The SAR analysis predicted an active generic structure, which correspond to potential compounds that would induce synthetic lethality in PME-1 depleted glioma cells (II Table 1). Interestingly, the identified active STS analogues also display a broad kinase inhibition profile and hence are referred to as multikinase inhibitors (MKI) (II Supplementary table 1).

5.1.2. PME-1 knockdown and multikinase inhibitors block colony formation ability of various GBM cell lines and GSCs (II)

The identified active MKIs were further evaluated for their ability to inhibit the colony growth of T98G, U251MG and U87MG-luc (luciferase expressing) human glioblastoma cell lines (II Figure 2B-C and Supplementary figure 2). In order to obtain meaningful and quantitative data a plugin tool for the freely available ImageJ software program was developed (I; described in section 5.2) and used for the automated analysis of colony formation assays performed in this study. In T98G cells, a small but significant reduction in colony growth was observed with PME-1 knockdown alone, however, treatment with MKIs resulted in a very drastic drop in the growth of PME-1 silenced cell colonies (II Figure 2B). Similar reduction in colony formation ability upon MKI treatment was observed in the PME-1-depleted U251MG and U87MG-luc cells, albeit at slightly higher concentration for some compounds (II Figure 2C and Supplementary figure 2).

In order to study the effect of PME-1 knockdown and MKI treatment on glioblastoma stem cells (GSCs), we acquired glioblastoma patient-derived cell lines established from the clinical samples cultured under stem-cell enriching conditions (on matrigel ECM coated flasks, in serum free media) (Ahmed et al, 2015; Fael Al-Mayhani et al, 2009). Two of these GSC cell lines, E2 and R10, were silenced for PME-1 expression and treated with STS or UCN-01. These combination treatments displayed significant reduction in the clonogenic growth of both GSC lines (II Figure 2E-F). These results validated the applicability of

synthetic lethal combination of PME-1 depletion and MKI treatment in GBM cells with genetically variable background.

Moreover, T98G cell clones with stable PME-1 knockdown were generated using lentiviral shRNAs (II Figure 6A). The colony formation potential of GBM cells with lower PME-1 expression was reduced upon treatment with MKIs, indicating higher sensitivity to the MKIs (II Figure 6B-C). These results suggested that the GBM patients stratified based on lower tumor PME-1 expression are likely to be sensitive to MKI monotherapies.

5.2. ImageJ plugin 'ColonyArea' for automated quantitative analysis of colony formation assays (I)

A bundle of java-based files and macros that performs automatic image processing and quantification of the scanned colony formation assay plate images was developed for an open-source image analysis software ImageJ, and named as 'ColonyArea' plugin (I Figure 1). The major tasks performed by the plugin are: 1) converting input image to 8-bit gray scale, 2) separate individual wells to create a stack of cropped wells, 3) based on the staining intensity calculate well-specific background threshold, 4) build a stack of wells with applied threshold, 5) calculate the colony area percentage and intensity percentage for individual wells (I Figure 1-4). The plugin provides two measurements of colony growth, 'area percentage' that represents the percentage of well area covered by cell colonies, and secondly 'intensity percentage' that in addition to area also consider the number of cells in the colonies (proportional to staining intensity). The ColonyArea plugin was used to calculate the IC50 of STS and UCN-01 in T98G cells by colony formation assay (I Figure 5A-B and Supplementary figure S1-S4). Both 'area percentage' and 'intensity percentage' estimates provided concurrent IC50 values for STS and UCN-01 in the lower nanomolar range, and in agreement with the previous reports (I Figure 5C). The performance of 'ColonyArea' plugin was in very good agreement with a previously used absorption based colony formation assay quantification method (Kueng et al, 2007) (I Figure 6). Therefore, ColonyArea provides a quick and reliable automatic quantification of the colony formation assays without the need for expensive equipment. It also eliminates the interpersonal variation (from manual counting), and preserves the samples for reanalysis and documentation compared to absorbance based measurements.

5.3. Depletion of PME-1 suppresses tumor growth in response to UCN-01 treatment in subcutaneous U87MG xenograft mouse models (II)

To test the described results *in vivo*, highly tumorigenic luciferase expressing U87MG cells (U87MG-luc) were used for generating tumors in athymic nude mice. Among active MKIs, UCN-01 was chosen based on its nanomolar concentration range efficacy in cell culture experiments (II Figure 2 and Supplementary figure 2). The first *in vivo* testing was carried out using U87MG-luc cells transiently transfected with scrambled (S) or PME-1 (P) siRNA *in vitro*, followed by subcutaneous implantation of cells on opposite mouse flanks performed one day after transfection. Once the palpable tumors appeared, mice received daily treatment with UCN-01 (intraperitoneal 3 mg/Kg body weight) for 7 days. Strikingly, UCN-01 treatment showed dramatic regression of the tumors with low PME-1 expression (P) as compared to control tumors (S) (with high PME-1 expression) within the same mice (II Figure 3A-D).

Another *in vivo* strategy involving intra-tumor siRNA treatment was employed to further evaluate the synthetic lethal combination therapy. Here, mice with subcutaneous U87MG-luc tumor xenografts were treated with intra-tumor Scr or PME-1 siRNA injection on alternate days, and intraperitoneal UCN-01 (daily for 10 days). This treatment strategy also displayed significant tumor suppression specifically in mice receiving PME-1 siRNA as compared to the Scr siRNA, and UCN-01 treatment (II Figure 3E-F).

5.4. PME-1 silencing reactivates 'specific' PP2A complexes required for multikinase inhibitor response in GBM (II)

Using PP2A-C immunoprecipitation based phosphatase activity assay, specific knockdown of PME-1 displayed enhanced PP2A activity in T98G cells (II Figure 4A). Moreover, PP2A inhibition by okadaic acid (OA) abolished the PME-1 siRNA and STS treatment mediated synthetic lethality in cell cultures (II Figure 4B). However, PME-1 regulates PP2A activity and substrate specificity by affecting the binding of certain regulatory B-subunits to the core dimer (Janssens et al, 2008). Therefore, it was relevant to identify those B-subunits that are required for PME-1 siRNA mediated MKI response. For this purpose, a panel of validated siRNAs against various PP2A B-subunits was purchased, and co-depletion of each B-subunit along with PME-1 was tested for their ability to abrogate STS treatment response (apoptosis). This analysis revealed that the inhibition of three specific B-subunits, namely R2A/PR55α, R5A/PR61α and R5B/PR61β, can abolish the PME-1 siRNA and STS mediated synthetic lethality (II Figure 4F and Supplementary figure 3C). In conclusion, PME-1 silencing reactivates R2A, R5A and R5B

B-subunit containing PP2A complexes in order to sensitize GBM cells to MKI treatment.

5.5. HDAC4 is a PME-1 dependent PP2A target mediating kinase inhibitor resistance in GBM (II)

In order to identify specific PME-1 regulated PP2A targets responsible for kinase inhibitor resistance in gliomas, the existing literature was studied to create a list of proteins that were known to interact with the R2A, R5A and R5B containing PP2A complexes (II Supplementary table S2). Among these potential PP2A targets, seven proteins were randomly selected. Some of these target proteins were inhibited by specific chemical inhibitors (MEK/ERK, PI3K/AKT, TGFBR and PIM kinase), whereas few others by siRNA (Myc, HDAC4 and NFkB), and their response to STS treatment was analyzed in T98G cells (II Figure 5A-B). Interestingly, whereas no synergistic apoptotic response was observed with any chemical inhibitor, or with NFkB depletion (II Figure 5A-B), depletion of Myc or HDAC4 phenocopied the PME-1 depletion-induced synthetic lethality (II Figure 5A). Surprisingly, downregulation of HDAC4 expression was observed in cells transfected with Myc siRNA (II Figure 5C), suggesting that the synthetic lethality seen in the Myc silenced cells was linked to HDAC4 downregulation. Therefore, HDAC4 was further validated as a potential PME-1 regulated PP2A target promoting kinase inhibitor resistance in GBM. Importantly, HDAC4 and PME-1 depleted cells showed very similar sensitivity profiles to different indolocarbazole analogs, both in nuclear fragmentation and in clonogenicity assays (II Figure 5D-E and Supplementary figures S4A-C). Similar to PME-1 silencing, the RNAi depletion of HDAC4 also inhibited the colony growth of GSCs in response to MKI treatment (II Supplementary figure 4A-E). Additionally, co-depletion of PME-1 and HDAC4 induced apoptosis as efficiently as depletion of either of them alone (II Figure 5F). These identical synthetic lethality response profiles strongly indicate that HDAC4 mediates PME-1-driven kinase inhibitor resistance in GBM. Importantly, co-depletion of PPP2R2A B-subunit, implicated in PME-1-depletion induced synthetic lethality (II Figure 4F) did not block synthetic lethality in HDAC4 depleted cells (II Figure 5I). This further indicates that PME-1 and PP2A function upstream of HDAC4. Furthermore, a proximity ligation assays (PLA) using rabbit anti-HDAC4 together with mouse anti-PP2A Cα-subunit antibodies was performed. PLA utilizes unique DNA oligonucleotide-tagged secondary antibody probes that recognize two different primary antibodies. The close proximity of probes results in DNA hybridization and amplification, producing a fluorescent signal at each protein-protein interaction site. The PLA performed in T98G glioma cells confirmed a physical association between HDAC4 and PP2A (II Figure 5H).

HDAC4 is a class IIa histone deacetylase that shuttles between the nucleus and cytoplasm, and has certain putative non-histone targets in both cellular compartments (Clocchiatti et al, 2013). Majority of HDAC4 expression has been detected in the cytoplasm, and in certain neuronal cells the cytosolic HDAC4 has been associated with increased cell survival (Chen & Cepko, 2009; Li et al, 2012). In T98G cells, immunostaining for HDAC4, and the proximity ligation assay confirmed that the expression of HDAC4 and its interaction with PP2A were mainly localized to the cytosolic cellular compartment (II Figure 5H and Supplementary figure 4G). Importantly, in human astrocytic glioma samples a similar cytosolic HDAC4 localization was observed (II Figure 6D). Further an antibody recognizing the general lysine acetylated proteins (ac-lys) was employed to demonstrate that the HDAC4 silencing upregulates the acetylation of certain cytosolic proteins (uncharacterized putative HDAC4 deacetylation targets) (II Figure 5J). Remarkably, a similar pattern of increased acetylated cytosolic proteins was observed in the PME-1 silenced cells (II Figure 5J). Thus, PME-1 expression appears to promote HDAC4 deacetylase activity possibly via PP2A inhibition.

Besides, the pan-HDAC inhibitors, panobinostat and romidepsin, which have been previously demonstrated to inhibit HDAC4 activity and even its protein expression (by promoting degradation) (Du et al, 2015; Fischer et al, 2015), displayed synthetic lethality in GBM cell lines in combination with STS or UCN-01 (II Figure 5K-N and Supplementary figure 4H-I). Interestingly, the HDAC inhibitor SAHA which inhibits HDAC4 only at very high (micromolar range) concentrations, did not synergize with MKIs (II Figure 5K and N). The concentrations of all three HDAC inhibitors resulted in increased histone H3 acetylation, however, the downregulation of HDAC4 expression was observed only with panobinostat and romidepsin, but not with SAHA (II Supplementary figure 4I). Thus, the downregulation of HDAC4 relates to HDAC inhibitor compound-mediated multi-kinase inhibitor response in GBM cells.

5.6. BAD expression is required for apoptosis induction by PME-1/HDAC4-silencing and multikinase inhibitor treatment (II)

Apoptosis triggered by various upstream regulators (in response to extrinsic or intrinsic signals) culminates at the activation of cysteine proteases called caspases (Ouyang et al, 2012). The effector caspases -3, -6 and -7 execute the cell death program by cleaving and/or destructing the vital cellular proteins (for example PARP and lamin A/C), organelles and DNA (Ouyang et al, 2012). In this study, the activity of the effector caspase-3 and -7 was measured in the T98G cells in response to PME-1 knockdown and STS treatment (II Figure 1E). As expected, a very high induction of the caspase-3/7 activity was noticed

upon STS treatment in PME-1 silenced cells. Next, by pre-treatment of the cells with pan-caspase inhibitor *z*-VAD-FMK, it was confirmed that the synthetic lethality mediated by the combination treatment is completely dependent on caspase activation (II Figure 1F).

It has been established in the apoptosis field, that different 'BH3 only' Bcl-2-family proteins (BAD, BID, BIM, NOXA and PUMA) act as mediators of distinct upstream regulatory pathways that lead to mitochondrial permeability, cytochrome *c* release and apoptosis (Czabotar et al, 2014). Among these, the pro-apoptotic protein BAD acts as a mediator between the upstream growth factor survival signaling (MAPK and PI3K signaling) and mitochondrial apoptosis, whereas other BH3-only proteins are either activated by death receptors signaling (BID and BIM) or by p53 (NOXA and PUMA) in response to DNA damage (Czabotar et al, 2014). Since both arms of the combination therapy proposed in this study target the survival pathways kinase signaling, BAD was hypothesized to be critical factor for apoptosis induction by the combination therapy. To this end, western blotting indicated that BAD expression is present in all GBM cell lines used in this study (data not shown). Moreover, the co-depletion of BAD in T98G cells abrogated the apoptosis induction by combined PME-1 siRNA and STS treatment (II Figure 1G). Similar blockage of synthetic lethality was also demonstrated by the HDAC4 siRNA (II Figure 5G). Therefore, the expression of BAD is required for the execution of apoptosis by the combined PME-1/HDAC4 inhibition and MKI treatment. However, BAD activation alone does not suffice for the synthetic lethal phenotype displayed by PME-1/HDAC4 depletion, as can be concluded by the absence of a synergistic activity between BCL-2/BCL-XL inhibitor, ABT263, and STS treatment (II Figure 1H).

In conclusion, BAD expression is required to sensitize GBM cells to MKI and PME-1 or HDAC4 depletion combination therapy, and its absence may lead to kinase inhibitor resistance.

5.7. PME-1, HDAC4 and BAD as potential stratification markers for predictive multikinase inhibitor therapy response in glioma patients (II)

The protein expression of HDAC4 and BAD was analyzed by immunohistochemisty (IHC) in grade II – IV human astrocytic glioma patient samples (n=139) in which the expression of PME-1 has been previously characterized (Puustinen et al, 2009). The expression of HDAC4 in the clinical glioma samples was mostly localized to the cytoplasm including the samples with low expression level (II Figure 6D). The HDAC4 expression showed a very significant direct correlation with the glioma tumor grade (II Figure 6D-E), similar to that reported for PME-1 in this cohort (Puustinen et al, 2009). Moreover, in the clinical glioma

samples the expression of HDAC4 was directly associated with the expression of PME-1 (II Supplementary figure 5B). Thus, the mechanistic crosstalk between PME-1 and HDAC4 identified in the cell culture experiments appears to exist also in the clinical glioma specimens.

Nearly half of the glioma samples in this cohort showed low to nonexistent expression for PME-1 or HDAC4, referred as PME-1^{low} or HDAC4^{low} (II Figure 6D). The analysis of BAD expression also showed a significant number of samples with no or low expression level (BAD^{low}) (II Figure 6D, F-H). Based on the findings of this study (described in section 5.6), the patients with moderate to high BAD tumor expression (BAD^{high}) are expected to respond to the MKI treatment, whereas the BAD^{low} tumor expression might predict for 'resistance' to these therapies (II Figure 6D, F-H). The correlation analysis between PME-1 or HDAC4 and BAD staining, revealed that about 17% of glioma patients displayed PME-1^{low} BAD^{high}, and 12% displayed HDAC4^{low} BAD^{high} tumor expression (II Figure 6F-G). Based on the results that tumors with low expression of PME-1 or HDAC4 are sensitive to MKIs, the PME-1^{low} BAD^{high} and HDAC4^{low} BAD^{high} serve as potential patient stratification markers for 'response' to MKI as monotherapy (II Figure 6H). Additionally, a significant percentage of patients (37%) displayed tumors with HDAC4^{high} BAD^{high} expression signatures (II Figure 6G). These glioma patients constitute a subgroup of potential 'responders' to the combined HDAC4 inhibitor and MKI therapy (II Figure 6H).

5.8. PME-1 expression as an independent prognostic marker of favorable outcome in colorectal cancer (CRC) (III)

5.8.1. Protein expression analysis of PME-1 in TYKS rectal cancer cohort

In order to study the expression of PME-1 in colorectal cancer (CRC), a commercially available PME-1 antibody was examined to confirm specific detection of PME-1 in CRC cell lines HCA-7 and CW-2, by using western blotting and immunofluorescence (IF) (III Figure 1). The tested antibody specifically recognized one band corresponding to PME-1 (44 KDa) by western blotting, which was absent in the PME-1 silenced cells (III Figure 1A). The IF analysis using this antibody visualized intense PME-1 expression in CRC cells (III Figure 1B and C). After confirming the specificity of PME-1 antibody, IHC staining was performed on 195 rectal cancer tumors isolated from patients diagnosed at the Turku University Central Hospital (TYKS) (III Figure 2A). Tumors were surgically removed from the patients, some of whom were preoperatively treated with long-course chemo/radiotherapy or short-course radiotherapy. The clinical characteristics of the rectal cancer patients are summarized in publication III Table 1. PME-1 staining indices were

calculated based on the 'staining intensity levels' (scored as -, +, ++ or +++), and the 'fraction of cells' in a sample with different staining intensity levels. The staining indices were scored from 0 to 3 (representative images shown in III Figure 2A). Among the studied clinicopathological variables, significant correlation of PME-1 staining index was found with gender and recurrent disease (III Table 2 and Supplementary table S1). Rectal cancer tumors from males displayed lower PME-1 staining as compared to females (III Table 2). Interestingly, a lower PME-1 expression strongly associates with the recurrence of rectal cancer ($p = 0.03$), suggesting PME-1 expression as a prognostic marker of recurrent disease (III Table 2).

5.8.2. High PME-1 expression predicts better survival of the CRC patients

The rectal cancer patients from TYKS cohort were separated into two groups based on the tumor PME-1 staining index: PME-1 high (index value above median), PME-1 low (index value below median). A correlation analysis by Kaplan-Meier estimates was conducted for the disease free survival (DFS) of patients and the PME-1 staining index. Surprisingly, in this univariate analysis, high PME-1 expression corresponded to longer DFS (116.7 months versus 110.7 months, $p = 0.007$) (III Figure 2B).

To confirm these findings, an independent CRC patient cohort available online from the cancer genome atlas (TCGA) database was used (COADREAD, n = 396) (TCGA, 2012). In this database, the PME-1 gene expression (*PPME1*) data from an RNA sequencing analysis was available (exon array IlluminaHiseq). The tumor *PPME1* mRNA expression (cutoff value -0.075 or median) was used to separate the samples with high or low *PPME1* expression (III Supplementary figure S1A-B). The univariate Kaplan-Meier survival analysis in the COADREAD cohort also revealed a similar correlation between high *PPME1* expression and longer overall survival (OS) of the patients (2213 days versus 1762 days, $p = 0.005$) (III Figure 2C and Supplementary figure S1C). Thus, PME-1 expression (mRNA and protein) predicts for a favorable CRC patient outcome in independent patient cohorts.

5.8.3. Multivariate analysis of CRC patients identified PME-1 expression as an independent prognostic factor

In order to inspect the role of PME-1 as a prognostic marker independent of the other confounding variables that can possibly influence the results of univariate survival analysis, a multivariate analysis using Cox proportional hazards regression model was conducted for both TYKS and COADREAD patient cohorts (III Table 3). The variables used for this

analysis were: sex, age (≤70 vs. >70 years), postoperative N (negative vs. positive), vascular invasion (negative vs. positive), circumferencial margin (≤2 vs. >2 mm), and PME-1 expression (high vs. low). Only those variables could be analyzed for which sufficient data was available for all the covariates. This analysis demonstrated that along with male gender and postoperative N (regional lymph node tumor cell) positivity, low tumor PME-1 expression was independent prognostic factor related to poor DFS and OS estimates for the CRC patients (III Table 3). In addition, tumor circumferencial margin (>2 mm), and old age (>70 years) predict poor DFS and OS respectively (III Table 1). Disease recurrence was a strong independent prognosticator for worse disease-specific survival (DSS) in TYKS rectal cancer patient cohort (III Table 1).

5.8.4. PME-1 knockdown does not affect CRC cell viability and survival signaling

Next, the role of PME-1 expression on cell proliferation and survival of CRC cells was studied. The siRNA-mediated knockdown of PME-1 was utilized to evaluate its effect on the viability of CRC cell lines, HCA-7 and CW-2 (III Figure 3A-B). Consistent with the results of survival analysis in CRC patients, and opposite to the previously reported oncogenic effects of PME-1 in glioblastoma and other cancers, the PME-1 depletion in CRC cells had no inhibitory effect on their proliferation or viability (III Figure 3A-B). In gliomas and other cancers depletion of PME-1 expression has been linked with PP2A activation and subsequent inhibition of the MAPK/ERK and/or PI3K/AKT pathways by reduced phosphorylation of the signaling proteins (Puustinen et al, 2009; Wandzioch et al, 2014). However, in CRC cells, PME-1 knockdown did not reduce phosphorylation of AKT and ERK; rather there was a trend towards increased phosphorylation of these survival-signaling proteins (III Figure 3C-D). Therefore, different from gliomas, endometrial and lung cancers, PME-1 appears to play an anti-survival role in CRC.

6. DISCUSSION

6.1. PME-1 drives kinase inhibitor resistance in human glioblastoma

PME-1 knockdown in GBM cells reduces viability and modestly inhibits colony growth without observable apoptosis, as previously reported and confirmed in this study (II) (Puustinen et al, 2009). Further, by two different drug-screening experiments, this study identified that PME-1 depletion sensitizes GBM cells to multikinase inhibitors by triggering massive apoptosis (II). Therefore, ablation of PME-1 in gliomas serves as 'apoptosis sensitizer' to the kinase inhibitors.

GBM has remained one of the most drug resistant cancers, especially towards the targeted kinase inhibitor therapies (Cloughesy et al, 2014; Lau et al, 2014; Reardon et al, 2014). Alterations in the pro-survival RTK/PI3K/AKT pathway, and cell cycle and apoptosis regulatory TP53 and RB pathways drive malignant progression in GBM (Brennan et al, 2013). The mechanisms mediating GBM resistance to RTK inhibitors have been under intense research; however, due to lack of potential clinical anti-glioma therapies targeting Ser/Thr kinases (e.g. enzastaurin for PKC), much less is known about the resistance mechanisms associated with this class of kinase inhibitors (Lau et al, 2014). The results of this thesis identified expression of PME-1 as potential mechanism responsible for GBM cell's resistance towards a number of different classes of kinase inhibitors (II). Notably, a significant apoptotic response in PME-1 depleted T98G cells was found for selective inhibitors of PKC/PKA (H7, enzastaurin, GÖ-6976, and K252c), PI3K (LY294002), cell cycle kinases CHK1/CDK1/2 (SB-218078), or multi-RTKs (sunitinib) (II). Though significant, these responses were less potent than those detected for multikinase inhibitors combined with PME-1 depletion (II). It seems plausible that the remaining activities of kinases operating in distinct cellular pathways or at different levels within the same pathway are responsible for the lower efficacy of the selective kinase inhibitors. It is a generally accepted view that the multi-targeted anti-cancer therapy strategies perform better than the monotherapies (Knight et al, 2010; Sathornsumetee et al, 2007; Wilson et al, 2014). In line with this notion, multikinase inhibitor PKC412 (midostaurin) that inhibits all four categories of kinases described above (PKC/PKA, AKT, CDK1, and several RTKs) displayed robust apoptosis of GBM cells in which PME-1 expression was inhibited (II). Similar response was demonstrated by STS, UCN-01, and CEP-701 (lestaurtinib) inhibitor treatment (II). Since these MKIs display different preferential kinase inhibition profiles (Karaman et al, 2008; Zarrinkar et al, 2009), further

studies are needed to identify key kinases whose inhibition is responsible for synergistic GBM cell death in the absence of PME-1. However, because a number of PKC/PKA selective inhibitors displayed apoptosis in PME-1 silenced cells, this family of kinases is expected to be a major contributor, synergizing with the PME-1 inhibition strategy.

PME-1 is an established negative regulator of PP2A function (Ogris et al, 1999; Puustinen et al, 2009; Xing et al, 2008). Importantly, this study elucidates that the reactivation of specific PP2A complexes by PME-1 depletion sensitizes the GBM cells to MKIs (II). This study proposes PP2A reactivation as a potential anti-GBM strategy. PP2A reactivation can be achieved by treatment with pharmacological PP2A activator compounds or by inhibition of the PP2A inhibitor proteins, such as PME-1, as identified in this study. In the light of limited clinical efficacy of MKIs such as sunitinib in GBM patients (Hutterer et al, 2014; Pan et al, 2012), PP2A reactivation strategies might circumvent the kinase inhibitor resistance by normalizing the aberrantly active phospho-signaling prevalent in cancers. These combinations might also lower the required MKI doses and reduce the toxic effects arising from the high doses needed due to their low efficacy as single-agent therapies (Hutterer et al, 2014).

Despairingly, the evaluation of the identified active MKIs (STS, UCN-01, CEP-701, K252a and PKC412) in intracranial animal models is restricted by the inability of these compounds to penetrate BBB. Coincidentally, new approaches have been developed that could enhance the delivery of small molecule inhibitors to brain tumors (Poon et al, 2016; Timbie et al, 2015; van Tellingen et al, 2015). Specifically, FUS (focused ultrasound) method could be used along with MKI-loaded microbubbles to deliver these potential anti-GBM agents to the specific locations where GBM tumor cells remain after surgical removal of the tumor mass. Interestingly, lipid encapsulation of STS has been reported to diminish its toxic effects *in vivo* (Mukthavaram et al, 2013; Tang et al, 2016). Systemically delivered STS-lipid-nanoparticles displayed preferential accumulation in tumors, and dramatic regression of subcutaneous U87MG tumors (in xenograft mouse model) (Mukthavaram et al, 2013), and multidrug resistant murine breast cancer cell line (EMT6/AR1) tumors in syngeneic BALB/c mouse model (Tang et al, 2016). Likewise, other MKIs can also be encapsulated in the nanoparticles, which can be delivered to GBM tumors by tagging them with BBB-penetrating peptides or using FUS (Nance et al, 2014; Timbie et al, 2015). Alternatively, the nanoparticles can be conjugated with peptides that specifically recognize brain tumor cells. For instance, a novel peptide has been identified that recognizes mammary-derived growth inhibitor (MDGI) protein expressed on the invasive brain tumor and tumor endothelial cells (Hyvonen et al, 2014). These strategies would promote the preclinical and possibly clinical evaluation of the identified MKIs in GBM.

6.2. PME-1 regulated PP2A activity mediates MKI response in GBM

Protein phosphorylation plays an important role in the cellular signaling, by transferring the survival or cell-death signals across the cellular machinery. Protein kinases and phosphatases regulate the phosphorylation status of thousands of proteins in a cell and thereby maintain a state of homeostasis necessary for normal cell function (Brautigan, 2013). However, in cancerous cells this homeostasis is disrupted by various molecular changes leading to an increased flux through the pro-survival signaling pathways, which promote malignant growth. On one hand, most of these alterations involve activation of the oncogenic protein kinases, transcription factors and anti-apoptotic proteins, on the other hand, the inhibition of tumor suppressor genes such as protein phosphatases and pro-apoptotic proteins (Hahn & Weinberg, 2002). Historically, PP2A was considered as a ubiquitous non-specific Ser/Thr protein phosphatase, until it was discovered that one of the many different B-subunits direct the PP2A activity to a specific set of target proteins (Slupe et al, 2011; Van Kanegan et al, 2005). The PP2A-C Leu309 methylation status and direct binding with PME-1, affect the preferential binding of certain B-subunits possibly at the expense of other B-subunits, while the binding of some B-subunits is not influenced (Janssens et al, 2008). My thesis study suggests that PME-1 inhibits PP2A complexes containing R2A/PR55α, R5A/PR61α and R5B/PR61β subunits in order to promote MKI resistance (II). Simultaneous depletion of one of these three B-subunits rescued the PME-1-depletion mediated MKI response, whereas the other tested B-subunits of R2/PR55 and R5/PR61 family did not influence this response (II). Interestingly, methylated Leu309 PP2A-C is an essential requirement for binding of R2/PR55 family B-subunits (Janssens et al, 2008; Sents et al, 2013), in line with our findings. However, we discovered a very distinct intraclass specificity among the members of both R2/PR55 and R5/PR61 family B-subunits for their ability to inhibit PME-1-depletion mediated MKI response. These results are particularly important because the genetic loss of these B-subunits in tumors might confer resistance to PME-1-depletion and MKI combined treatment. A very significant proportion of breast (~40%) and prostate (~67%) cancer cases have been reported to contain deletions of *PPP2R2A* (R2A/PR55α subunit) (Cheng et al, 2011; Curtis et al, 2012). Reduced expression of R5A/PR61α has been associated with more aggressive metastatic stage of melanomas (Mannava et al, 2012). In this regard, GBM appears to be the best-case scenario for PP2A reactivation anti-cancer strategy, because most of the PP2A subunits are genetically intact in this cancer, particularly *PPP2R2A* deletion could be detected in <1% of GBM cases in all available cancer repositories (Brennan et al, 2013) (www.cbioportal.org). However, in one study the protein expression of PP2A-Aα subunit was found to be

lower in a significant fraction of gliomas in the absence of any genetic defects (Colella et al, 2001). Therefore, it is possible that the PME-1 depletion might not reactivate PP2A in these tumors. It is also possible that these tumors do not overexpress PME-1 because of other PP2A inhibitory mechanisms operational in these tumors. Nevertheless, these possibilities need to be evaluated in such tumors. The mechanisms inhibiting PP2A-Aα expression in these tumors could provide alternative therapeutic targets for malignant gliomas.

The specificity of MKI response in GBM for PME-1-regulated PP2A activity was also reflected by the observation that CIP2A-depletion or SET inhibitor FTY720 treatment failed to show a similar synthetic lethality with MKIs (II). The likely explanation for these results could be that CIP2A and SET inhibit a subset of PP2A (B-subunit) complexes different from those regulated by PME-1, and it is the inhibition of PME-1 regulated PP2A pool that is responsible for MKI resistance in GBM. However, these results do not exclude the possibility that the other PP2A inhibitory proteins may have potential oncogenic or drug resistance promoting functions in GBM. As a matter of fact, elevated expression of CIP2A protein has been found in 55.6% of grade I-IV astrocytic glioma tumor samples, along with a strong correlation of its expression with tumor aggressiveness (Yi et al, 2013). The mRNA expression of *SET* has been reported to be higher in malignant brain tumors compared to normal tissues using oncomine database analysis (Westermarck & Hahn, 2008). FTY720 inhibits invasion of GBM stem cells (GSCs) and potentiates the anti-glioma response of TMZ in intracranial GSC mouse model (Estrada-Bernal et al, 2012). However, the anti-cancer effects of FTY720 can also be influenced by the inhibition of S1P pathway, which is the major target of FTY720 activity (Patmanathan et al, 2015). Interestingly, the anti-glioma effects of FTY720 reported by Estrada-Bernal et al were not regulated by either of these two pathways, indicating the existence of other possible, yet unknown, functional targets of FTY720 in gliomas (Estrada-Bernal et al, 2012). Since ~50% of the astrocytic glioma tumors are PME-1 positive and about 55% are CIP2A positive (Puustinen et al, 2009; Yi et al, 2013), it can be hypothesized that PP2A inhibition in gliomas can occur by inhibition of either one of these two (proto)oncoproteins and possibly also others like SET. Whether there is a mutual exclusivity between the expression of PME-1 and CIP2A in gliomas remains a very interesting question to be studied.

Paradoxically, PP2A inhibition also leads to cancer cell death. Therefore, the focus of some research groups has been on the pharmacological inhibition of PP2A as an anti-glioma strategy (Gordon et al, 2015; Lu et al, 2009). This paradoxical function of PP2A is related to its function in DNA damage repair (DDR) and cell-cycle checkpoint signaling. Under

normal conditions, PP2A complexes containing R2A/PR55α, R5E/PR61ε and R5C/PR61γ B-subunits dephosphorylate several DDR and cell cycle checkpoint proteins such as pRb, CHK1/2, ATR, p53 and γ-H2AX, and promote cell-cycle recovery once the DNA repair has been completed (Shaltiel et al, 2015). Inhibition of PP2A impairs DDR resulting in accumulation of DNA double strand breaks (DSBs), impaired cell cycle checkpoints, and pre-mature entry into the cell cycle (Chowdhury et al, 2005; Shaltiel et al, 2015; Yan et al, 2010). The PP2A-inhibitor anti-cancer strategies rely on the cancer cell death by mitotic catastrophe resulting from the continued mitotic entry with accumulating DNA damage lesions and chromosome segregation defects (Gordon et al, 2015). However, genetic instability, such as that instigated by PP2A inhibition, is an enabling characteristic of hallmark cancer capabilities (Hanahan & Weinberg, 2011). Accumulating DNA lesions may give rise to clones of cancer cells that survive the mitotic catastrophe and PP2A inhibition may exaggerate their survival leading to tumor recurrence. Nevertheless, a phase 1 clinical trial has started to evaluate PP2A inhibitor LB1 (LB100) in combination with docetaxel in solid tumors (www.clinicaltrials.gov). The results of this trial would shed light on these possible consequences.

In contrast, this thesis study suggested that reactivation of a subset of PP2A complexes can sensitize GBM cells to MKI therapy (II). This strategy is therefore based on the normalization of tumor suppressor PP2A function and induction of programmed cell death when aberrantly active oncogenic kinases are simultaneously inhibited. A defined cellular context based on PME-1 or HDAC4 and BAD expression outlines the usability of this approach as GBM therapy (discussed later). The additional evaluation of R2A/PR55α, R5A/PR61α, R5B/PR61β and PP2A-Aα subunit expression can be added to further streamline the patient subgroups most likely to benefit from this therapy.

6.3. HDAC4 subcellular localization and function in GBM

This study identified HDAC4 as a PME-1 dependent PP2A target, whose inhibition phenocopies the PME-1 depletion mediated MKI response (II). HDAC4 is a nucleo-cytoplasmic shuttling protein that is heavily phosphorylated on multiple sites (Clocchiatti et al, 2011; Di Giorgio & Brancolini, 2016; Mielcarek et al, 2015). Its phosphorylation by various Ser/Thr kinases promotes binding to the 14-3-3 protein, which (and by other possible mechanisms) sequesters it to the cytosolic compartment (Di Giorgio & Brancolini, 2016). Conversely, the dephosphorylation, proteolytic processing and other modifications that expose its NLS promote its import into the nucleus (Di Giorgio & Brancolini, 2016). In this context PP2A mediated dephosphorylation of HDAC4 leads to its

nuclear translocation (Illi et al, 2008; Kozhemyakina et al, 2009; Paroni et al, 2008).

The role of subcellular HDAC4 fractions in malignant and normal cell survival has been controversial. In retinal neurons, 'cytosolic' HDAC4 promotes survival and inhibits natural apoptosis, partially by stabilization of HIF1α (Chen & Cepko, 2009). On the other hand, 'nuclear' HDAC4 was shown to inhibit CDK1 and cell cycle progression to protect the cerebellar granule neurons (CGNs) from low potassium (LK) stress, and neuroblastoma HT22 cells from oxidative stress (Majdzadeh et al, 2008). Contrary to these, other researchers have reported that the LK stress leads to CGN cell apoptosis via 'nuclear' accumulation of HDAC4 and repression of its targets MEF2 and CREB-dependent transcription (Bolger & Yao, 2005). Similar transcriptional repression by nuclear HDAC4 in addition to the loss of cytosolic HDAC4 has been linked with neurodegeneration in ataxia telangiectasia (AT) patients as well as in ATM -/- mice (Li et al, 2012). A limitation of these functional studies related to HDAC4 localization has been an almost exclusive use of exogenously expressed wild-type, various mutant or deletion HDAC4 constructs, adding a hint of uncertainty to the existence of similar functions under physiological conditions. Nevertheless, a commonality between all reported studies including validation at the endogenous protein level, is the predominantly 'cytosolic' localization of HDAC4 in the brain tissues and cultured cells of CNS origin (Chen & Cepko, 2009; Darcy et al, 2010; Fitzsimons, 2015; Li et al, 2012). Thus, it can be concluded that the CNS cell viability is associated with the cytosolic HDAC4 expression, though it is possible that the altered HDAC4 subcellular localization as a whole (both cytosolic loss and nuclear accumulation) work in conjunction to mediate the growth-suppressive and/or cell death promoting functions (Fitzsimons, 2015).

In line with the previous reports, HDAC4 was found to be predominantly cytosolic also in the cultured GBM T98G cells as well as in the clinical astrocytic glioma tumor samples in this study (II). The interaction between PP2A and HDAC4 was also detected mainly in the cytoplasm (II). HDAC4 silencing increased the lysine-acetylation of certain unidentified proteins in the cytoplasm, reflecting the reduced HDAC4 deacetylase activity (II). Strikingly, a very similar cytosolic lysine-acetylated protein pattern could be detected in PME-1 silenced cells, suggesting these proteins to be regulated by PME-1/PP2A/HDAC4 pathway (II). Characterization of the pro-survival cytosolic HDAC4 targets in GBM is an intriguing area for future research.

The synthetic lethality response of HDAC4 silencing with MKIs was remarkably similar to the PME-1 depletion in GBM cells (II). Simultaneous depletion of PME-1 and HDAC4 did not enhance the apoptosis induction by silencing either of these two proteins, suggesting

that they function in the same pathway in this phenotype (II). In the astrocytic glioma patient tumor material, expression of PME-1 exhibited strong correlation with HDAC4, confirming this crosstalk in the clinical context (II). HDAC4 phosphorylation at Ser298 has been identified by mutagenesis studies (using exogenous HDAC4 expression) to be regulated by PP2A (Paroni et al, 2004). The unavailability of a phospho-Ser298-HDAC4 antibody prevented the analysis of this phosphorylation in this thesis study. The evaluation of phosphorylation at Ser246 and Ser632 of HDAC4, using specific antibodies, did not reveal any changes in PME-1 silenced T98G cells (data not shown). However, based on the results that PME-1-regulated PP2A activity inhibits HDAC4 deacetylase activity in the cytoplasm, the precise location where HDAC4 interacts with PP2A, it is highly likely that these proteins function in the same pathway (II). The identity of PME-1/PP2A regulated phosphosite(s) on HDAC4 and/or other possible mechanisms by which PP2A regulates cytosolic HDAC4 activity needs further investigation.

Importantly, this study also identified HDAC4 as a GBM oncoprotein, whose expression is associated with the astrocytic glioma tumor progression (II). HDAC4 siRNAs or chemical inhibitors enhance the GBM cell apoptosis in combination with MKIs. Interestingly, an acquired platinum-resistance in ovarian cancer has been associated with HDAC4 overexpression, deacetylation-mediated STAT1 activation and its nuclear translocation (Stronach et al, 2011). Additionally, HDAC4 expression in breast cancer cell lines imparts resistance to 5-FU chemotherapy, which involves histone deacetylation and transcriptional repression of SMAD4 and possibly other genes of TGFβ pathway (Yu et al, 2013). This thesis study extends the therapy resistance role of HDAC4 in gliomas, which is mediated by deacetylation of still to be identified cytosolic proteins. Furthermore, the pan-HDAC inhibitors, panobinostat and romidepsin that inhibit HDAC4 activity and expression (most likely by ubiquitination and degradation as previously reported), have been granted FDA approval for treatment of multiple myeloma and T-cell lymphoma (Frye et al, 2012; Laubach et al, 2015). Both inhibitors are BBB-penetrable, and panobinostat has been tested in glioma clinical trials. The results of a phase-2 trial of panobinostat in patients with recurrent HGGs showed no benefit of its addition to bevacizumab, although panobinostat was well tolerated in the patients (Lee et al, 2015a). Based on the results of this thesis, we propose that the HDAC inhibitors might perform better as anti-glioma agents in combination with multikinase inhibitor drugs.

6.4. Patient stratification in gliomas

With the advent of genomic, transcriptomic and proteomic profiling studies in a large panel of patient samples, it has become clear that the

individual tumors display distinct molecular patterns, with some alterations occurring more frequently than others. The presence of certain molecular alterations may define sensitivity (response) to some therapies, whereas resistance to some other therapies. Therefore, now-a-days personalized medicine is regarded as the future of anti-cancer therapies. Moreover, it is critical to identify the possible drug resistance mechanisms within the patients, so as to specifically direct a therapy to only those patient subgroups that have the higher chance of benefiting from each therapy.

In the present study, it was shown that the inhibition of a pro-apoptotic protein BAD abolish the apoptotic GBM cell death mediated by PME-1 or HDAC4 depletion combined with MKI treatment (II). Thus, loss of BAD expression instructs GBM cell resistance to PME-1 or HDAC4 inhibition therapy. Notably, the patient stratification signatures based on PME-1 or HDAC4 and BAD tumor expression described a significant proportion of patients who can benefit from MKI monotherapy (PME-1^{low} BAD^{high} or HDAC4^{low} BAD^{high}) or combined therapy with MKI and HDAC inhibitors (HDAC4^{high} BAD^{high}) (II). These results might form the basis of potential human glioma clinical trials using MKI therapies in the future. This information might also provide a rationale for the previously conducted glioma trials with kinase inhibitors that failed to show clinical benefit in an unsorted patient population. For instance, the IHC evaluation of these stratification markers from the tumor material of glioma patients treated with enzastaurin could be evaluated to confirm these findings (Wick et al, 2010; Wick et al, 2013).

6.5. Mechanisms regulating survival signaling in colorectal cancer

In this thesis, the expression and significance of PME-1 was also studied in CRC (III). In addition to the astrocytic gliomas (Puustinen et al, 2009), overexpression of *PPME1* mRNA (~83%) and protein has been previously reported in a small panel of endometrial cancers (Wandzioch et al, 2014). *PPME1* gene amplification with associated PME-1 overexpression has been found in about 3-4% of gastric and lung cancers (Li et al, 2014). PME-1 expression in gliomas and endometrial tumors associates with an increasingly malignant state (Puustinen et al, 2009; Wandzioch et al, 2014). The knockdown of PME-1 expression in various cancer cells reduces PP2A-C Leu309 demethylation, which in turn promotes PP2A-mediated dephosphorylation and inhibition of MEK/ERK and AKT signaling pathways (Jackson & Pallas, 2012; Li et al, 2014; Puustinen et al, 2009; Wandzioch et al, 2014). PME-1 silencing also suppresses the cell proliferation, viability, anchorage-independent growth and/or invasion of these cancer cells under different conditions. Thus, PME-1 acts as an oncoprotein in gliomas, endometrial, lung and gastric cancers.

In the light of these growth-promoting functions of PME-1 in various cancers, it is an intriguing finding that in CRC, a high expression of PME-1 correlates with less recurrent disease and longer patient survival (III). The PME-1 expression analysis at both mRNA and protein level from two independent patient cohorts with almost 200 or more patients per cohort provided convincing evidence of the existence of the unexpected role of PME-1 in CRC (III). Interestingly, the PME-1 knockdown in CRC cell lines failed to show any reduction in the phosphorylation of ERK and AKT pro-survival proteins, or the cell viability (III). It raises the possibility that the PME-1/PP2A or PP2A/target circuit might be broken in these cells, and/or PME-1 may have PP2A independent functions that are responsible for its peculiar function in CRC (Figure-7).

Given that PP2A inhibition is an essential requirement for malignant transformation of human cells, the presence of other PP2A inactivation mechanisms may abolish the PP2A regulatory function of PME-1 (Figure-7). For instance, somatic mutations or homozygous deletion of *PPP2R1B* (PP2A-Aβ subunit) occur in 8-15% of CRCs, altering its interaction with PP2A-C subunit, and inhibiting overall PP2A activity (Takagi et al, 2000; Tamaki et al, 2004; Wang et al, 1998). Among the B-subunits regulated by PME-1 in human gliomas, repressed protein expression of R2A/PR55α subunit was detected in ~42% of CRC tumors (n=21) (Cristobal et al, 2014a). Another member of the R2 family B-subunits which are highly sensitive to PME-1 regulated PP2A-C methylation, *PPP2R2B* (R2B/PR55β) is epigenetically silenced in a very significant (>90%) number of CRCs by promoter hypermethylation (n=24) (Tan et al, 2010). At the post-translational level, the PP2A-C subunit is highly phosphorylated at Tyr307 residue in CRCs (~31%, n=35) (Cristobal et al, 2014b). This phosphorylation has been associated with the lack of Leu309 methylation and the inability of R2A/PR55α subunit binding to the dimeric PP2A complex (Yu et al, 2001). These genetic and molecular alterations collectively account for a very large fraction of CRCs where PP2A function is inhibited via PME-1-independent mechanisms (Figure-7). Additionally, a new function has been recently proposed for PME-1 in mouse embryonic fibroblasts, wherein it protects PP2A-C from proteasome degradation (Yabe et al, 2015). Whether this function could promote PP2A activity due to stabilized expression in 'high' PME-1 CRC cells is also a topic of further research (Figure-7).

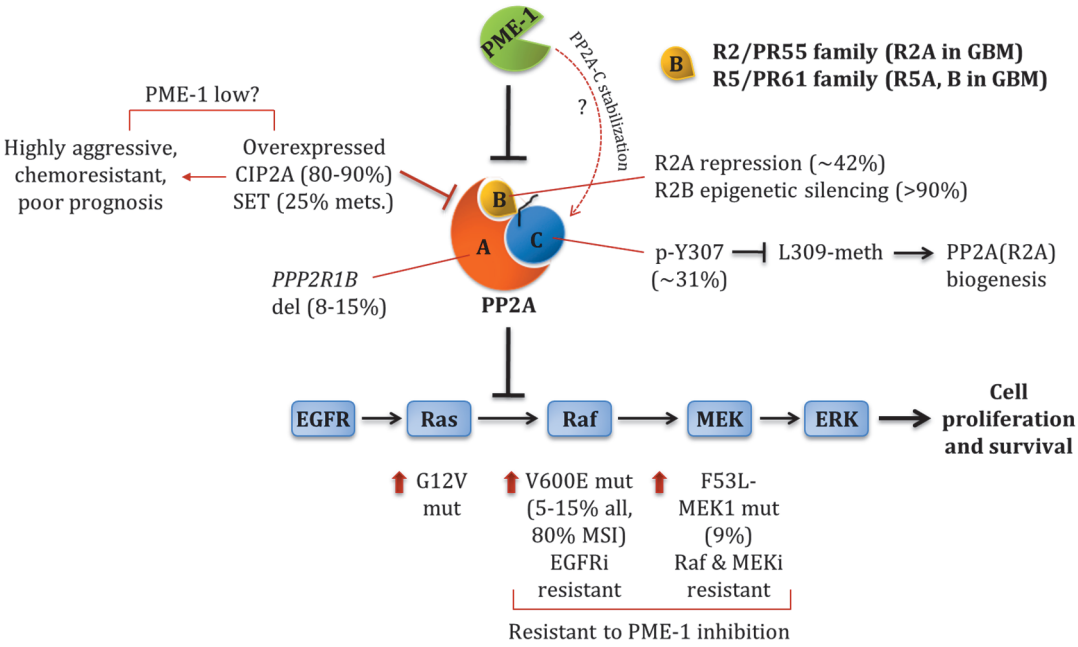


Figure-7. Illustration of possible mechanisms responsible for PME-1 independent survival signaling in CRC. *Black lines indicate the PME-1 regulated PP2A function in Ras/Raf/MEK/ERK pathway as reported in other cancers including gliomas and GBM. Red lines indicate known alterations in CRC, and their reported frequency of occurrence in parenthesis. L309-meth – methylated PP2A-C Leu309; mut – mutation; del – deletion; mets. – metastatic CRC.*

Furthermore, the expression of other PP2A inhibitory proteins may account for other major subgroups of CRC tumors, which possibly do not require PME-1 expression in order to inhibit PP2A (Figure-7). In line with this notion, CIP2A overexpression is demonstrated in a majority of CRCs (80 to >90%), and a high CIP2A expression correlates with worse patient prognosis in this disease (Bockelman et al, 2012; Junttila et al, 2007; Teng et al, 2012; Wiegering et al, 2013). Elevated levels of SET protein were detected in 25% of metastatic CRC tumor samples, and associated with drastically reduced patient survival (Cristobal et al, 2015). In these studies, both CIP2A and SET expression were linked with chemotherapy resistance in CRC cells. Based on the highly aggressive nature of the CIP2A and SET expressing CRC tumors, it can be postulated that the tumors with 'high' CIP2A or SET expression might not require PME-1 expression (PME-1 low) to inhibit PP2A activity (Figure-7). On the other hand those with 'low' CIP2A or SET might benefit from the PME-1 expression (PME-1 high) as a contributing factor to tumor progression. Therefore, it is possible that the better survival observed for 'high' PME-1 expressing CRC patients in this study is due

to 'low' CIP2A or SET expression. It remains a tempting hypothesis to be evaluated in the future with careful simultaneous analysis of various PP2A inhibitory proteins in the CRC tumor samples.

Another possibility for the unexpected role of PME-1 in CRC could be the existence of aberrant signaling downstream of PP2A (Figure-7). In gliomas, PME-1 promotes Ras/Raf/MEK/ERK pathway activity by inhibiting the PP2A function at a level upstream of Raf (Puustinen et al, 2009). Mutations which lead to constitutive activation of K-Ras (G12V) and B-Raf (V600E) are commonly found in CRC, and often associate with resistance to EGFR inhibitor therapies and poor prognosis (Lech et al, 2016). In AKT expressing HEK-T cells transformed with the H-Ras^{G12V} mutant, PME-1 knockdown suppressed ERK phosphorylation and inhibited cell proliferation (Puustinen et al, 2009). However, the replacement of H-Ras^{G12V} with B-Raf^{V600E} mutation in this model displayed resistance to the PME-1 silencing mediated growth inhibition effects. The oncogenic B-Raf^{V600E} mutation occurs in 5-15% of all CRCs, and ~80% of the tumors which display microsatellite instability (MSI) (Thiel & Ristimaki, 2013). Moreover, mutations in MEK1 (F53L mutation in 9% tumors) have been shown to promote CRC resistance to combined Raf/MEK inhibitor therapies (Ahronian et al, 2015). These alterations might contribute to the constitutive downstream ERK pathway signaling, and insensitivity of CRC cells to PME-1 regulated functions (Figure-7).

The mechanisms responsible for the function or dysfunction of PME-1 in CRC certainly demand thorough research efforts. Nevertheless, a favorable prognostic role of PME-1 in CRC could be useful to identify the patients at lower risk of recurrence, so as to protect them from the highly toxic chemotherapies. Importantly, this is the first study to report on the association of PME-1 expression with patient survival in any human cancer. Future research might uncover the cancer-specific clinical and biological relevance of PME-1 and other PP2A inhibitory proteins.

7. SUMMARY

PME-1 inhibits tumor suppressor PP2A activity in order to promote tumor growth and progression in human gliomas. This study showed for the first time that PME-1 loss sensitizes GBM cells to kinase inhibitor therapies resulting in a synthetic lethal apoptotic response (Figure-8-1.). The GBM cell response to multikinase inhibitors required reactivation of PME-1-regulated specific PP2A complexes containing PPP2R2A/PR55α, PPP2R5A/PR61α and PPP2R5B/PR61β B-subunits. This study also identified PP2A target HDAC4 to be regulated by PME-1. HDAC4 loss or pharmacological inhibition reproduced the MKI response demonstrated by PME-1 depletion (Figure-8-2.). Importantly, the expression of BAD appeared to be necessary for the apoptosis induction by identified synthetic lethal combinations (Figure-8-3.). This study also defined stratification signatures based on the tumor expression of PME-1, HDAC4 and BAD to stratify potential glioma patients likely to benefit from MKI monotherapy or combined HDAC inhibitor and MKI therapy. As a whole, this study suggests PP2A reactivation as a strategy to circumvent kinase inhibitor resistance in GBM.

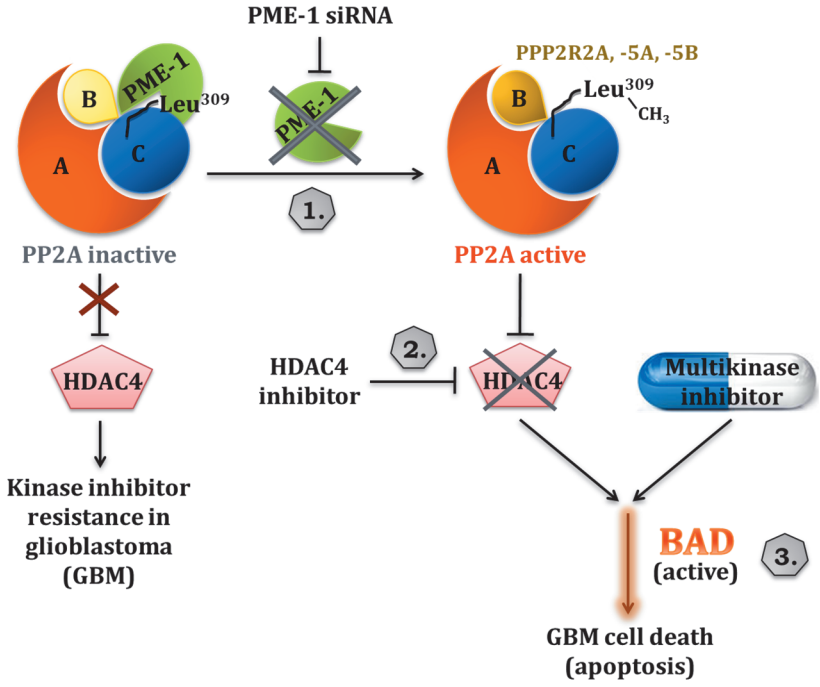


Figure-8. PME-1-driven kinase inhibitor resistance pathway in glioblastoma (GBM) and strategies for its inhibition. *(1.) PME-1 inhibition reactivates PP2A and sensitizes GBM cells to multikinase inhibitors (2.) HDAC4 inhibition as an alternative strategy for kinase inhibitor response in GBM (3.) The apoptosis induction by PME-1 or HDAC4 inhibition and multikinase inhibitor combination strategies require expression of pro-apoptotic protein BAD.*

This study is also the foremost to report that PME-1 is an independent prognostic factor in CRC. A high PME-1 mRNA and protein expression predicted for a better patient outcome and less recurrence in CRC. The conventional PME-1 regulated pro-survival signaling was found to be dysfunctional in the CRC cells.

As a technical advance, this thesis reports on the development of an image analysis tool, ColonyArea, to facilitate the automated quantification of large colony formation assay datasets.

8. ACKNOWLEDGEMENTS

I extend my warm acknowledgements to many great people whose support and guidance made this thesis possible and my journey as a doctoral candidate an enjoyable experience.

First and foremost I express my deepest respect and profound gratitude to my supervisor Professor Jukka Westermarck. Your constant encouragement and guidance kept me motivated throughout my doctoral dissertation. Thank you for giving me the freedom to work, for believing in me and for being an inspiration to me.

I sincerely thank previous and current director of Turku Centre for Biotechnology (CBT), Professor Riitta Lahesmaa and Professor John Eriksson, for providing excellent research facilities and an inspiring atmosphere. Professor Olli Carpén is thanked for accepting me as a doctoral candidate in Department of Pathology at University of Turku. I feel privileged to be part of two graduate schools. Turku Doctoral Programme of Biomedical Sciences (TuBS) and Turku Doctoral Programme of Molecular Medicine (TuDMM) are acknowledged for providing financial support, and for organizing excellent scientific and recreational activities. I especially thank Professor Olli Lassila, Docent Kati Elima, Nina Widberg and Eeva Valve for planning graduate school events and keeping all practical things running smooth. I also thank TuBS for supporting my travel to several scientific conferences abroad, including Cold Spring Harbor Laboratory, USA and Hindsgavl, Denmark to name a few. A big thanks to Outi Irjala for skillful administrative assistance in the final stages of my dissertation.

I greatly acknowledge the critical evaluation of my thesis by Docent Marko Hyytiäinen and Professor Veerle Janssens. I thank Professor Frank Furnari for accepting the invitation to be my opponent. I am thankful to my thesis committee members Professor John Eriksson and Docent Cecilia Sahlgren for steering my thesis progress.

I express my immense gratitude to Docent Jari Sundström and Docent Daniel Abankwa for the opportunity to work with them in collaboration, and all the co-authors especially Docent Hannu Haapasalo, Camilo Guzmán, Manish Bagga, Adam Elzagheid, Xi Qiao, Oxana Denisova, Mikael Jumppanen, Emilia Peuhu, Professor Anthony Chalmers and Professor Pirjo Laakkonen for their contribution and guidance.

The administration and technical staff, and core services at CBT deserve a special mention for their persistence in keeping things running smoothly. Thanks to Aila Jasmavaara, Sirkku Grönroos, Ulla Karhunen, Susanna Pyökäri, Pasi Viljakainen, Hannele Vuori, Virpi Korpiranta, Anneli Takala, and Mårten Hedman. CBT cell imaging core, viral vector

core and microarray and sequencing centre personals especially Jouko Sandholm, Markku Saari, Ketlin Adel and Artur Padzik are acknowledged for their sincere help.

I also thank all the present and past members of the Westermarck group who helped to create a lively work atmosphere. Thank you Anni Laine for never losing your nerve on my many (several times silly) questions; Eleanora Mäkelä née Sittig for being the 'coolest dude' in the group; Inga Pukonen for not just excellent technical help but also for being a caring friend; Otto Kauko for sharing your excitement for science and help with all matters related to statistics; Xi Qiao for long discussions on cooking and our shared Asian roots; the most eligible biochemist Juha Okkeri being the 'fun element' and for joining me in the late-at-lab division; Taina Kalevo-Mattila for your help with so many things and always-ready attitude; Tiina Arsiola for being the leading-lady in the house, and for giving your 100% in keeping things in order; Christian Rupp for your encyclopedia of technical knowledge especially on antibodies; Oxana Denisova for bringing new energy to the glioma-division; Mikael Jumppanen for your contribution from a chemist's perspective; Karolina Pavic for your sparkling introduction to novel techniques; Joni Merisaari, Laxmana Yetukuri and Srikar Nagelli for some cherished moments during my final year. Among the past members I thank Minna Niemelä, Leni Kauko, Tuuli Halonen, Christophe Côme and Anna Lipsanen for their help during many needed times during these years. Anni, Joni and Tiina are also greatly acknowledged for Finnish translation of my thesis abstract.

I also thank Docent Pannu Jaakkola, Professor Johanna Ivaska and their research group members for sharing lab and office space, and for creating inspiring surroundings. Other research groups at CBT, especially Professor Riitta Lahesmaa's group are acknowledged for sharing research material and equipment for my thesis work.

I am grateful to my friends in Finland and in India for their support during all these years. Simply said, my lunch breaks would have been boring without sharing food and thoughts with some of you. A really warm thanks to Yashu and Raghu for all the adventurous trips, your support during critical moments in my life, and little Lasya for bringing joy. Thanks to Poonam, Subhash, Prasanna, Amruta, Santosh, Preeti, Justyna, Artur and Ania for the many lovely get-togethers, crazy late night gaming sessions and so many memorable moments. Special thanks to Mohit, Shishir and Tuuli for helping me survive the cold and dark Turku (arriving from a pleasantly warm Indian climate), and make it my second home. I owe my vote of thanks also to Megha and the Diwali party organizers for all the lively evenings and festivities here in Turku.

My heartiest thanks goes to my family for making me what I am today and for believing in me. Thank you mummy, papa, Jassi and Harry for your unconditional love and support. I also thank my grandparents, Bebe, Nani and Nana ji, whose foresight and values paved the way to my privileged education. My journey to doctorate would have been incomplete (probably would not even have started) without you, Hasan. Thank you for standing by my side in this long and bumpy journey.

This work was financially supported by TuBS and TuDMM doctoral programmes, Jane and Aatos Erkko foundation, Turku University Foundation, University of Turku Faculty of Medicine grant, Cancer Society of Finland, Orion Research Foundation, Instrumentarium Science Foundation and K. Albin Johanssons Foundation.

Turku, June 2016

Amanpreet Kaur

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